Diagnostic accuracy of C-reactive protein and procalcitonin, compared with composite reference standards, to discriminate between tuberculosis, *Pneumocystis jirovecii* pneumonia and bacterial pneumonia in HIV-infected inpatients meeting WHO criteria for seriously ill.

By

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DECLARATION

I, *Fiona Mendelson*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Signature Signed by candidate

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Abstract

Tuberculosis, bacterial community-acquired pneumonia (CAP), and *Pneumocystis jirovecii* pneumonia (PJP) are the commonest causes of hospitalisation in HIV-infected individuals. Prompt diagnosis and treatment initiation are important to reduce morbidity and mortality, but are hampered by overlap in clinical and radiological presentation and limited diagnostic resources in resource-poor settings. Simple, affordable tests are needed for developing improved clinical algorithms. C-reactive protein (CRP) and procalcitonin have shown diagnostic utility for respiratory tract infections, however few studies have focussed on their ability to distinguish between tuberculosis, CAP, and PJP in adult HIV-infected inpatients.

We performed a cross-sectional study on secondary data to evaluate the diagnostic accuracy of C-reactive protein (CRP) and procalcitonin compared to composite reference standards, to discriminate between the three target infections in HIV-infected inpatients. Participants had been recruited in two district level hospitals in Cape Town, South Africa, admitted with current cough and danger signs in accordance with the WHO algorithm for tuberculosis in seriously ill HIV-infected patients. Study clinicians were blinded to CRP and procalcitonin results and laboratory staff who performed biomarker tests on stored serum were blinded to patient diagnoses.

248 participants met study criteria and case definitions: 133 with tuberculosis, 61 with CAP, 16 with PJP, and 38 with mixed infection. Elevated CRP was found in 98% of participants. The cutoff used to guide antibiotic initiation in lower respiratory tract infections correctly identified 82% of those with CAP, 84% with tuberculosis and 50% with PJP. The differences in median CRP and procalcitonin concentrations between the three infections were statistically significant, but distributions overlapped considerably, and CRP and procalcitonin cut-offs with sensitivities of $\geq 90\%$ were found for all three target infection pairs, however corresponding specificities were low. Receiver operating characteristic areas under the curve for CRP and procalcitonin were between 0.68 and 0.74 for PJP versus tuberculosis and PJP versus CAP.

CRP and procalcitonin showed limited value in discriminating between the three target infections due to widely overlapping distributions, but diagnostic accuracy was higher for discriminating PJP from CAP or tuberculosis. Our findings suggest that CRP and procalcitonin may have greater diagnostic utility as part of a panel of biomarkers or in clinical prediction rules.

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PART A: Research Protocol

Diagnostic accuracy of C-reactive protein and procalcitonin, compared with composite reference standards, to discriminate between tuberculosis, *Pneumocystis jirovecii* pneumonia, and bacterial pneumonia in adult HIV-infected inpatients meeting WHO criteria for seriously ill.

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Diagnostic accuracy of C-reactive protein and procalcitonin, compared with composite reference standards, to discriminate between pulmonary tuberculosis, *Pneumocystis jirovecii* pneumonia and bacterial pneumonia in adult HIV-infected inpatients meeting WHO criteria for seriously ill.

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1. Summary

HIV-related tuberculosis (TB), bacterial community-acquired pneumonia (CAP) and *pneumocystis jirovecii* pneumonia (PJP) are major causes of hospitalisation, particularly in low and middle income countries with high HIV disease burden. Lack of optimal diagnostic algorithms adds to the challenge of differentiating between the three infections and delays initiation of appropriate treatment. This study seeks to explore the diagnostic accuracy of two non-specific inflammatory biomarkers: C-reactive protein and procalcitonin that have shown some promise for discrimination between HIV-related respiratory infections of different aetiologies. To date, their performance is incompletely defined and further studies are needed to evaluate their potential as part of diagnostic algorithms.

2. Background

Respiratory infections in HIV-infected people are a major cause of hospitalisation (1), and mortality and morbidity are negatively influenced by delayed treatment or initiation of inappropriate therapy as a result of diagnostic delay (2).

Defining the aetiology of respiratory infection in HIV patients remains a significant challenge as there is considerable overlap in the clinical and radiographic presentation of the three commonest respiratory infections: pulmonary tuberculosis, bacterial community-acquired pneumonia, and *Pneumocystis jirovecii* pneumonia. Utility of current diagnostic methods is hampered by resource constraints in low and middle income countries, and poor performance of available laboratory diagnostic methods (3-5). There is an on-going search for effective and affordable alternative diagnostic measures to improve patient outcomes.

Patient management is guided by a widely used clinical screening algorithm: the World Health Organization algorithm for the diagnosis of tuberculosis in seriously ill patients presenting with severe symptoms of respiratory infection The recently 2016 updated version (6) introduced some improvements to the previous 2007 algorithm, however there remains an absence of effective triage

tests to assist clinicians in determining who needs further more complex diagnostics recommended by the algorithm, such as Xpert MTB/RIF, or who requires empiric PJP treatment.

Improved diagnostic algorithms employing assays suitable for low-resource settings would help to prevent delay to diagnosis and initiation of treatment, and avoid inappropriate treatment or unnecessary. C-reactive protein and procalcitonin are two non-specific inflammatory biomarkers that have been identified as having potential value as triage tests in algorithms for patients with respiratory illnesses. A number of studies have explored diagnostic value of C-reactive protein for ruling out tuberculosis, particularly in HIV-infected patients with smear-negative disease (7,8), whilst to date, the main utility of PCT testing for respiratory infections in non-HIV infected populations has been to rule-out bacterial infection and to aid appropriate antibiotic prescribing (9).

We conducted a systematic literature search for studies involving HIV-infected individuals with two or more of the target diseases and C-reactive protein and/or procalcitonin testing. This search yielded few studies and fewer still that reported diagnostic accuracy measures. Only two studies reported diagnostic accuracy for C-reactive protein in all three infections, with conflicting results. One case notes review found poor specificity in discriminating between the three (10), whereas the other found higher specificity (82%) in discriminating CAP from the other respiratory infections, however CRP was combined with another biomarker and participant selection included hospital acquired respiratory infections and other mycobacterial infections beside tuberculosis (11). A South African study (12) found good sensitivity for both biomarkers in discriminating CAP from tuberculosis, although selection was for participants with suspected CAP and may mean that tuberculosis was underrepresented in analyses. We only identified one study for procalcitonin that found statistically significant differences in concentrations between each of the three infections, however no diagnostic accuracy analyses were reported (13).

3. Rationale

This study is designed to explore the diagnostic accuracy of C-reactive protein and procacitonin in seriously ill adult HIV-infected inpatients with suspected respiratory infection, towards determining whether either have potential utility as a triage test in a diagnostic algorithm. This study is related to a larger main study aimed at improving current algorithms for managing tuberculosis.

For this purpose, we propose a secondary analysis of the main dataset: a large prospective HIV-infected cohort, recruited from two secondary level hospitals in Cape Town South Africa, serving high HIV and TB prevalence communities.

4. Research Question and Objectives

4.1 Research Question:

What is the diagnostic accuracy of C-reactive protein and procalcitonin, compared with composite reference standards, to discriminate between tuberculosis, *Pneumocystis jirovecii* pneumonia and bacterial community-acquired pneumonia in adult HIV-infected inpatients meeting criteria for seriously ill?

4.2 Primary objective:

To determine diagnostic accuracy of C-reactive protein and procalcitonin in predicting presence or absence of each of the three target infections.

4.3 Secondary objectives:

i. To determine optimal concentration cutoffs for C-reactive protein and procalcitonin for discriminating between those with and without each of the three target infections

ii. To determine to what extent C-reactive protein and procalcitonin concentrations differ between each target infection

5. Methods

5.1 Study design

This is a cross sectional diagnostic accuracy study, using secondary analysis of prospective cohort data. Our objectives are closely linked to those of the main study, which was designed to determine an evidence base for the development of improved algorithms in the management of seriously ill HIV-infected patients with tuberculosis (14). This study will comply with STARD guidelines (15).

5.2 Population and sampling

5.2.1 Population

Recruitment for the main study took place at two secondary level hospitals in Cape Town South Africa, serving over-lapping high HIV and tuberculosis prevalence communities. 195 patients were recruited from GF Jooste District Hospital from November 2011 to March 2013 and, following the closure of that hospital, 289 patients from Khayalitsha District Hospital from March 2013 to October 2014.

5.2.2 Method of sampling for the main study

Consecutive enrolment took place daily over the study period. Inclusion criteria were as follows: adult patients (aged 18 years and over), admitted to either study site, with cough of any duration and one or more danger signs defined in the WHO screening algorithm: respiratory rate >30/min, fever > 39^{o}C, pulse rate > 120/min and unable to walk unaided. All new medical admissions were screened for

study eligibility by the study research worker and study doctor.

Inclusion criteria were:

- Aged 18 and over
- HIV-infected (documented or confirmed on admission)
- Current cough of any duration with one or more WHO danger signs
- Sputum sample obtained

Exclusion criteria were:

- Failure to meet inclusion criteria
- Failure to consent
- History of tuberculosis and use of anti-tuberculosis therapy: either current use, completed within the previous month or defaulted treatment in the past 6 months
- Exacerbation of congestive congestive cardiac failure or chronic obstructive pulmonary disease
- Failure to provide a spontaneous or induced sputum specimen using an ultrasonic nebulizer and hypertonic saline

5.2.3 Participant selection for this study

Our study involves a sub-set of 388 of the original 500 participants patients enrolled from March 2012 to October 2014: 69 patients from GF Jooste District Hospital and 261 patients from Khayalitsha District Hospital. Funding for biomarker assays only became available after the start of the main study therefore participants enrolled prior to March 2012 were excluded. Our assumption is that the only difference between the main and sub-sets is enrollment date and does not pose a risk of bias.

Of the 388 patients, 248 met case definitions for one or more of the three target diseases and 10 patients had other definitive diagnoses, (case definitions are described in full in Section 6.3) . We excluded 58 patients who could not be categorised due to missing radiological data and 72 patients who did not meet case definitions.

Diagnoses of excluded patients

Of the remaining 82 patients, 10 had confirmed diagnoses other than tuberculosis, CAP or PJP and 72 were empirically diagnosed with one or more of the three target diseases but did not meet case definitions. Analysis of these 72 as 'probable cases' was rejected due to high prevalence of dual infection.

10 patients with confirmed diagnoses other than the target diseases were as follows:

- 2 patients with laboratory confirmed meningitis (1 with non-tuberculous mycobacterium)
- 1 patient had laboratory confirmed disseminated *Cryptococcus*
- 1 patient had laboratory confirmed emmonsia
- 1 patient had laboratory confirmed disseminated *Cryptococcus* and emmonsia
- 4 patients had bronchitis
- 1 patient had post-TB bronchiectasis with a pneumothorax

72 patients who did not fulfill case definitions were as follows:

- 26 patients with an empirical diagnosis of tuberculosis (and negative cultures) who were commenced on anti-tubercular treatment. 20 of these had coinfection with another pathogen.
- 59 patients with an empirical diagnosis of CAP (15 coinfected with TB)
- 4 patients with empirical diagnosis of PJP, (2 coinfected with TB)

Diagnoses of participants meeting case definitions

248 patients met one or more of the three target disease case definitions: 164 patients with tuberculosis, 98 with CAP, 24 with PJP; and 38 with mixed infection: 30 were coinfected with tuberculosis and CAP, 1 with tuberculosis and PJP, and 7 with CAP and PJP. Participants with more than one of the three target infections will be excluded from primary analysis as it is impossible to determine the contribution of each disease to the index test results. However, this unavoidably reduces our sample size.

5.2.4. Power calculation

Software used: STATA version 13.0 (StataCorp Inc. College Station, Texas, USA).

Power calculation for objective 1:

Using our fixed sample size consisting of single case-defined infections, we explored precision to detect 90% sensitivity for each biomarker for each of the three target infections versus the other two, with a proposed aim of ± 10% variation in 95% confidence intervals. Since we are evaluating performance of C-reactive protein and procalcitonin as triage tests, we selected sensitivities rather than other measures and minimum 90% was selected from WHO recommendations (16) . Our power calculation was limited by the availability of relevant studies reporting diagnostic measures. Our criteria for selection was any study reporting sensitivity for either biomarker in discriminating any of the three target infections in HIV-infected adults (8, 12, 17).

Expected sensitivities for each target infection were restricted to C-reactive protein as among studies evaluating procalcitonin in HIV-infected individuals with respiratory infections, none reported diagnostic measures. We explored a range of sensitivites with confidence interval of binomial proportions of 95% and 99%, using the Wilson-score interval, which is suited to smaller sample sizes (18). Since our data was not normally distributed, a second calculation was made using 85% of the original sample sizes as suggested by Lehmann (19). We estimated 95% CIs of 90% sensitivity to be 83%-94% for tuberculosis, 79%-96% for CAP, and 69%-99% for PJP. The small sample size for PJP accounted for wide 95% confidence intervals.

Power calculation for objective 2:

We explored power to detect mean differences between PCT and the three target diseases and between CRP and the three target diseases by estimating power for a two-sample means test, assuming unequal variances. We considered significance levels of 0.05 and 0.10 and 100% and

85% sample sizes. Estimations for procalcitonin were based on one study (13) with HIV-infected individuals with suspected CAP that reported mean effect sizes (with wide 95% CIs).
For C-reactive protein, we were only able to base calculations on studies reporting means and standard deviations for CRP in non-HIV infected individuals with tuberculosis or CAP (20-22).
Taking 80% as an accepted minimal power and alpha 0.05 and 85% sample size as our most conservative estimate, our study is adequately powered to detect minimum procalcitonin mean concentration differences of 50% between tuberculosis and PJP, 62% between CAP and PJP, 62% between CAP and tuberculosis and minimum C-reactive protein mean concentration differences of 36% between tuberculosis and PJP, 14% between CAP and PJP and 14% between CAP and tuberculosis.

6. Measurements

6.1 Types of measurements

- Laboratory and microbiological analyses
    - Three sputum samples on admission were sent for Gram stain, culture, and sensitivity, auramine staining for acid-fast bacilli (AFB), liquid mycobacterial culture and Xpert MTB/RIF
    - Blood samples on admission were sent for mycobacterial culture, full blood count, CD4 count and haemoglobin concentrations
    - Stored serum was used for C-reactive protein and procalcitonin assays, and β-D-glucan
    - Extra pulmonary samples (for example pleural fluid) were sent for mycobacterial culture
- Radiological: Chest x-rays were performed on admission and reviewed by a specialist radiologist, who was blinded to diagnoses and laboratory investigation results. An

experienced ultrasonographer performed an abdominal ultrasound within three days of admission to detect extra pulmonary or disseminated tuberculosis.

- Clinical: On admission, the study doctor carried out complete patient history and physical examination and oxygen saturation measurements.

6.1 Biomarker assays

A sample of blood was obtained from each participant on admission. Once clotted, samples were centrifuged and spun at 3500rpm for 10 minutes. The separated serum was decanted into sterile tubes, labelled with the patient's initials and study number and stored in a refrigerator at minus 70°C. For the purpose of this study stored serum from participants was analysed retrospectively. C-reactive protein and procalcitonin results had no role in patient referral or management and laboratory staff were blinded to patient diagnosis and outcome. Laboratory–based assays were performed at the National Health Laboratory Service (NHLS), Charlotte Maxeke Johannesburg Academic Hospital and the following assays were used:

- C-reactive protein using Siemens Advia 1800. Normal range <10mg/L and assay range 4-[304-336] mg/L
- Procalcitonin (sensitive) using Siemens Advia Centaur XP. Normal range for adults <0.02µg/L and assay range 0.02-75µg/L

6.2 Reference standards

Case definitions for each diagnosis were as follows:

Tuberculosis

a. postitive *Mycobacterium tuberculosis* culture from any site; AND
b. at least one symptom consistent with tuberculosis (cough, fever, night sweats, weight loss)

Community-acquired bacterial pneumonia, taken from Scott et al. (23).

a. cough ≤ 14 days; AND

b. one or more additional respiratory symptoms: sputum, breathlessness, chest pain, haemoptysis, or fever; AND

c. radiological evidence of pulmonary consolidation (confirmed by a radiologist)

***Pneumocystis jirovecii* pneumonia**, adapted from Centre for Disease Control (24). Full reference case definition and rationale for modifications are provided in table 1.

a. cough ≤ three months (84 days); AND

b. radiological evidence of diffuse bilateral interstitial infiltrates (confirmed by a radiologist); AND

c. oxygen saturation ≤ 92%

Table 1: CDC reference case definition for PJP and rational for modifications

	CDC case definition in the absence of laboratory confirmation	Case definition for this study	Rationale for modification
a.	History of dyspnoea on exertion or nonproductive cough of recent onset (within the past 3 months); AND	Cough ≤ three months (84 days); AND	Participants were recruited for main study with cough of any duration
b.	Chest x-ray evidence of diffuse bilateral interstitial infiltrates or gallium scan evidence of diffuse bilateral pulmonary disease; AND	Radiological evidence of diffuse bilateral interstitial infiltrates (confirmed by a senior radiologist); AND	Expert radiological opinion was applied to all study participants to stregthen case identification
c.	Arterial blood gas analysis showing an arterial pO_2 of <70mmHg or a low respiratory diffusing capacity (<80% of predicted values) or an increase in the alveolar-arterial oxygen tension gradient; AND	Oxygen saturation ≤ 92%	Pulse oximetry was performed on all patients on admission. Senior study clinicians determined 92% as a conservative proxy for pO_2 70mmHg (25)
d.	No evidence of a bacterial pneumonia		Only patients who are not co-infected (ie only meet one of the three study case definitions are included in primary analysis

7. Analysis Plan

7.1.Statistical software

STATA version 13.0 (StataCorp Inc, College Station, Texas, USA) will be used for analysis.

7.2.Baseline variables

The cohort will be described using the variables in table 2. For biomarker analyses, C-reactive protein and procalcitonin values below the detectable limit (BDL) of the assay will be substituted with half BDL (in preference to substitution with the assay limit or with zero, both of which have been shown to bias parameter estimates (26) . Summary statistics will be provided for overall measurements and for above detectable limit.

Table 2: Baseline variables

Variable	Type of variable	Measurement
Independent variables		
Age	Continuous	Mean & SD or median & IQR
Gender	Categorical binary	0/1 Male/Female
Diagnosis	Categorical: binary or nominal depending on analysis	Case-defined tuberculosis, CAP, PJP, co-infections, other infections & non-case-defined empirical diagnoses
CD4 count	Continuous	Mean & SD or median & IQR
White cell count	Continuous	Mean & SD or median & IQR
Haemoglobin concentration	Continuous	Mean & SD or median & IQR
β-D-glucan	Continuous	Mean & SD or median & IQR
Antiretroviral therapy (ART) status	Categorical binary	0/1 Currently not taking ART/taking ART
Antibiotic prophylaxis on admission	Categorical binary	0/1 Not taking prophylaxis/taking prophylaxis
Antibiotic therapy commenced prior to index test measurements	Categorical binary	0/1 Not commenced/commenced
Cotrimoxazole prophylaxis prior to admission	Categorical binary	0/1 Not taking prophylaxis/taking prophylaxis
Heart rate>120 b/m	Categorical binary	0/1 Over 120 or ≤ 120
Respiratory rate >30/min	Categorical binary	0/1 Over 30 or ≤ 30
Temperature > 39^{o}C	Categorical binary	0/1 Over 39 or ≤ 39
Inabilty to walk unaided	Categorical binary	0/1 walked in unaided or not
Dependent variables		
C-reactive protein	Numerical	Median & IQR
Procalcitonin	Numerical	Median & IQR

7.3. Outcome measures

Participants with more than one of the three target infections will be excluded from diagnostic analysis as it is impossible to determine the contribution of each disease to biomarker concentrations.

However, this unavoidably reduces our sample size, which is too small for generating a training set. However, appropriate cross-validation methods will be applied to the data.

Outcome measure 1:

Logistic regression will be used to generate receiver operating characteristic curves (ROC) and area under the curve (AUC) estimates for C-reactive protein and procalcitonin for each target infection versus the other two and for each target infection pair.

Outcome measure 2:

ROC analysis will be used to estimate sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratios and diagnostic odds ratios and AUCs (with 95% confidence intervals) for selected cutoffs for C-reactive protein and procalcitonin for each target infection versus the other two and for each target pair.

Outcome measure 3:

Our biomarker data is not normally distributed, therefore we will estimate median concentrations plus inter-quartile range (IQR) for C-reactive protein and procalcitonin for target infection. Differences in biomarker medians between the three infections will be analysed using the Kruskal-Wallis test, and between infection pairs using the Wilcoxon-Mann-Whitney test.

8. Ethics

The University of Cape Town Human Research Ethics Committee granted ethical approval for the main study HREC REF: 334/2011, renewal date 30/05/2016 (Appendix I).

8.1. Informed Consent

Participant consent was obtained by the study research worker, in English or Xhosa according to participant preference, filed with the source documents and checked for completion by the study doctor. Consent was deferred in any participants admitted in a confused state until an orientated condition was restored. Participants were able to withdraw from the study at any time.

8.2 Confidentiality

On enrolment, participants were de-identified and coded using a study number and own initials. All clinical information and investigation results were recorded on case report forms, stored in a dedicated folder in an on-site locked filing cabinet. Any corrections to the documents were signed and dated. Data was entered no less than weekly onto the password-protected database and backed up. Chest x-rays were digitally recorded, de-identified and stored on the study computer. Source documents will be stored by the principal investigator for a minimum of five years after the completion of the study and digital x-ray images retained.

8.3. Patient benefits and harms

This analysis poses no risk or discomfort to participants, as it will not involve further human subjects research. Blood serum used for biomarker assays was obtained from blood samples taken as part of routine care; all tests performed were part of standard diagnostic investigations, or for study as potential improvements to treatment algorithms. C-reactive protein and procalcitonin were done on stored serum in a batch after the study, therefore these tests had no role in patient management. Comparison reference standards comprised investigations that were also part of standard care.

All participants received routine standard of care, including appropriate investigations, clinical

review and initiation of antimicrobial therapies as per national guidelines. The admitting medical team was responsible for overall care and patient management.

For those whose HIV status was unknown or unconfirmed, HIV testing was performed as per national protocol. On discharge, access to antiretroviral therapy via local community clinics, appropriate to the individual patient, was organized.

Post-discharge clinical review was arranged on day 28 and 56 from enrolment to assess clinical response and outcome; this was brought forward if clinically indicated. Participants were reimbursed with R150 for attending the 28-day clinic follow-up appointment. The admitting medical team arranged readmission or further follow-up as necessary. Participants attending community TB/HIV clinics were provided with their final tuberculosis culture results to take to their clinics.

9. Stakeholders/dissemination of results

Stakeholders are patients at risk of the three target diseases, particularly in resource constrained settings with high HIV and TB prevalence and the clinicians and policy-makers involved in their care. Plans to distribute findings include publication and conferences.

10. Conflict of interest

No conflicts of interest exist.

11. Budget

No overhead costs are associated with this secondary analysis.

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Appendix 1: Ethics approval documents (University of Cape Town)

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G16.68
NGSH

Dear A/Prof Mendelson

PROJECT TITLE: ***An evidence-based algorithm for diagnosis of tuberculosis in HIV patients***

Thank you for submitting your new study to the Faculty of Health Sciences Human Research Ethics Committee.

DATE OF MEETING: 29 JULY 2011

DECISION: This study was approved at a full HREC meeting held on the 29 July 2011. The study is approved until **29 July 2012.**

Voting for approval was as follows: Approved 7 of the 12 core or nominated alternate members present; not approved 0; Abstentions 0.

Approved:
Research Protocol
NIH grant Application

Approval is granted for one year until 29 July 2012.

Please amend the consent form to make it easier for participants to understand. Re-imbursement should be given to participants and the amount stated in the consent form.

Please send us an annual progress report (website form FHS 016) if your research continues beyond the approval period. Alternatively, please send us a brief summary of your findings so that we can close the research file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

lemjedi

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

lemjedi

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

28 October 2016

HREC REF: 773/2016

Prof G Maartens
Clinical Pharmacology
K45, OMB

Dear Prof Maartens

PROJECT TITLE: COMPARED TO CURRENTLY USED COMPOSITE REFERENCE STANDARDS FOR SERIOUSLY ILL HIV-INFECTED ADULT INPATIENTS WITH PULMONARY TUBERCULOSIS, *Pneumocystis jirovecii* PNEUMONIA OR BACTERIAL COMMUNITY ACQUIRED PNEUMONIA (Masters Candidate - Ms F Mendelson) Sub-study linked to 334/2011

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th October 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period. (Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

The HREC acknowledge that the student, Fiona Mendelson will also be involved in this study.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☐ Approved	Annual progress report	Approved until/next renewal date	30.11.2018
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	13/11/2017

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	09 November 2017		
HREC REF Number	334/2011	Current Ethics Approval was granted until	30/5/2016
Protocol title	An evidence-based algorithm for diagnosis of tuberculosis in HIV patients		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	☐ Yes ✓ No		
If yes, could you please provide the HREC Ref's for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study			
Principal Investigator	Prof Marc Mendelson		
Department / Office Internal Mail Address	Division of Infectious Diseases & HIV Medicine		

1.1 Does this protocol receive US Federal funding?	✓ Yes	☐ No
1.2 If the study receives US Federal Funding, does the annual report require full committee approval?	☐ Yes	✓ No
1.3 Has sponsorship of this study changed? If yes, please attach a revised summary of the budget.	☐ Yes	✓ No

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

2. List of documentation for approval

3. Protocol status (tick ✓)

☐	Open to enrolment
☐✓	Closed to enrolment (tick ✓)
☐	Research-related activities are ongoing
☐	Research-related activities are complete, long-term follow-up only
✓	Research-related activities are complete, data analysis only
☐	Main study is complete but sub-study research-related activities are ongoing
☐	Study is closed → Please submit a Study Closure Form (FHS010)

4. Enrolment

Number of participants enrolled to date	500
Number of participants enrolled, since last HREC Progress report (continuing review)	66
Additional number of participants still required	NA

5. Refusals

Total number of refusals (participants invited to join the study, but refused to take part)	34

6. Cumulative summary of participants

Total number of participants who provided consent	500
Number of participants determined to be ineligible (i.e. after screening)	1554
Number of participants currently active on the study	0
Number of participants completed study (without events leading to withdrawal)	500
Number of participants withdrawn at participants' request (i.e. changed their mind)	0
Number of participants withdrawn by PI due to toxicity or adverse events	0
Number of participants withdrawn by PI for other reasons (e.g. pregnancy, poor compliance)	0
Number of participants lost to follow-up. Please comment below on reasons for loss of follow-up.	37
Participants moved away and/or unable to contact participants on telephone number or address supplied	
Number of participants no longer taking part for reasons not listed above. Please provide reasons below:	0

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

7. Progress of study

Please provide a brief summary of the research to date including the overall progress and the progress since the last annual report as well as any relevant comments/issues you would like to report to the HREC:
Last participant follow up ended 28/11/2014

8. Protocol violations and exceptions (tick ✓ all that apply)

✓	No prior violations or exceptions have occurred since the original approval
☐	Prior violations or exceptions have been reported since the last review and have already been acknowledged or approved
☐	Unreported minor violations that have occurred since the last review, as well as significant deviations not yet reported, are attached for review

9. Amendments (tick ✓ all that apply)

✓	No prior amendments have been made since the original approval
☐	Prior amendments have been reported since the last review and have already been approved
☐	New protocol changes/ amendments are requested as part of this continuing review (See note below)

Note: If new protocol changes are being requested in this review, please complete an amendment form (FHS006).
Specific changes in the amended protocol and consent/assent forms must be **bolded**, *italicised* or tracked and all changes must include a rationale.

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

10. Adverse events

10.1 Please provide below or attach a narrative summary of serious adverse events and/ or unanticipated problems since the last progress report. Please indicate changes made to the protocol and informed consent document(s) as a result (if not already reported to the HREC). Please comment on whether causality to any study procedure or intervention could be established.
None

10.2 Have participants received appropriate treatment/ follow-up/ referral when indicated (e.g. in the case of abnormal or incidental clinical findings, distress or anxiety)?		
☐ Yes	☐ No	✓ Not applicable
If yes, please describe:		

11. Summary of Monitoring and Audit Activities (tick ✓)

11.1 Was this study monitored or audited by an external agency (e.g. MCC, FDA)?		
☐ Yes	✓ No	☐ Not applicable

11.2 Did a Data and Safety Monitoring Board publish a report?		
☐ Yes	☐ No	✓ Not applicable

11.3 If yes, please identify the agency and attach a summary of the findings.					
Agency Name		Report attached	☐ Yes	☐ No	☐ Not applicable
		DSMB report attached	☐ Yes	☐ No	☐ Not applicable

11.4 Has there been any agency, institutional or other inquiry into non-compliance in this study, or any finding of non-compliance concerning a member of the research team?	
☐ Yes	✓ No
If yes, please explain:	

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

12. Level of risk (tick ✓)

12.1 In light of your experience of this research, please indicate whether the level of risk to participants has:	
☐	Increased
✓	Decreased
☐	Shown no change
If there has been a change, please explain:	

12.2 Please provide a narrative summary of recent relevant literature that may have a bearing on the level of risk.
N/A

13. Statement of conflict of interest

Has there been any change in the conflict of interest status of this protocol since the original approval? (tick ✓)	
☐ Yes	☐ No
If yes, please explain and if necessary attach a revised conflict of interest statement (Section #7 in the New Protocol Application Form FHS013):	

14. Signature

My signature certifies that the above is complete and correct.			
Signature of PI		Date	9th November 2017

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

PART B: Background

Evaluation of the role of C-reactive protein and procalcitonin in the diagnosis of respiratory infections in HIV-infected individuals.

Evaluation of the role of C-reactive protein and procalcitonin in the diagnosis of respiratory infections in HIV-infected individuals.

Student: Fiona Mendelson (MNDFIO001)

Supervisor: Professor Gary Maartens[1]

Co-supervisors: Dr. Molegobeng Ragaka,[1,2] Professor Andrew Boulle[3]

[1]Department of Medicine, University of Cape Town

[2]Department of Infection & Population Health, University College London

[3]Centre for Infectious Disease Epidemiology Research, School of Public Health and Family Medicine, University of Cape Town

1. Introduction

HIV- related respiratory infections are a major cause of hospitalisation globally, particularly in low and middle-income countries (LMICs) with high HIV disease burden. (1)

Defining the aetiology of respiratory infection in HIV patients remains a significant challenge as there is considerable overlap in the clinical and radiographic presentation of the three commonest respiratory infections: pulmonary tuberculosis, bacterial community-acquired pneumonia (CAP), and *Pneumocystis jirovecii* pneumonia (PJP). Mortality and morbidity in HIV-related pulmonary infections is negatively influenced by delayed treatment or initiation of inappropriate therapy as a result of diagnostic delay (2); poorly performing diagnostic methods, resource constraints and long waiting times for results hamper utility of current management algorithms. There is an on-going search for effective and affordable alternative diagnostic measures to facilitate appropriate treatment. The focus of our study is to evaluate the possible potential of two biomarkers as triage tests for adult HIV-infected inpatients presenting with symptoms of respiratory infection of unknown aetiology.

2. Burden of disease

Tuberculosis is a major global health burden. In 2015, the World Health Organization (WHO) estimated 10.4 million new cases and 1.8 million deaths, approximately a fifth being in HIV-infected individuals (3). Overall fatality rates showed wide geographical variation with the highest in the WHO African Region, which the report attributed to sub-optimal diagnostic tools and treatment services. (3). A systematic review of causes of hospital admission among people living with HIV worldwide found that tuberculosis accounted for 18%, and 27% of deaths (1).

In the same study, bacterial pneumonia accounted for 15% of hospital admissions and 17% of deaths. (1) CAP may occur at any stage of HIV infection, but more commonly at CD4 counts less than 200 cells/µl. (2). Causative organisms are the same as in the non-HIV infected population and include common Gram-positive bacteria, aerobic Gram-negative bacilli and atypical pathogens. (2). A global

review of CAP (4) found comparable incidence of atypical organisms across all regions of 20-28%. Although Asia and Africa were not as well represented as other regions, the review reported low antibiotic coverage for atypical pathogens in Asian and African settings, resulting in longer hospital stays and higher mortality.

PJP caused by the fungus *Pneumocystis jirovecii* is a major opportunistic infection in HIV-infected persons; prevalence varies, due in part to geographical differences in prophylaxis use and effectiveness (5). In the Ford et al. systematic review, PJP was responsible for 8% of admissions and 13% of deaths (1).

3. Diagnosis of HIV-related pulmonary infection

3.1 Current diagnostic methods

Differentiation between tuberculosis, CAP and PJP is not straightforward. In HIV-infected persons, atypical and ambiguous radiological presentation and overlapping clinical features pose an additional challenge to distinguishing between these three infections (6, 7). Furthermore, patients with tuberculosis and HIV have higher rates of smear-negative and extra pulmonary disease, compounding diagnostic difficulties by reducing performance of commonly used diagnostic measures for detecting tuberculosis (8, 9).

Aetiological diagnosis generally relies on laboratory methods to identify the causative organism, however each type of diagnostic test has disadvantages. In LMICs, diagnosis of PJP generally rests on radiological and clinical findings (10). Microscopy for *Pneumocystis* pneumonia requires induced sputum or bronchoalveolar lavage, obtained by invasive procedures; it is also expensive and seldom used in resource-constrained settings, whilst expectorated sputum has prohibitively poor sensitivity (10).

Sputum culture for CAP is subject to contamination by upper respiratory flora and both sputum and blood culture have been shown to have poor sensitivity and limited use in guiding patient management (11-13). *Mycobacterium tuberculosis* culture is not always available in low-resourced

settings and can take several weeks to process, contributing to delayed diagnosis (14); liquid media culture is faster than solid but prone to contamination (15). In addition, culture-based diagnostics are costly and challenging for resource-limited settings with poor laboratory infrastructure (16).
Sputum smear microscopy remains the most commonly available diagnostic test in resource-limited settings and has a high specificity but low sensitivity due to reduced bacillary load associated with HIV infection (17). Polymerase chain reaction (PCR)-based Xpert MTB/RIF is more sensitive than smear microscopy, although sputum-smear negative tuberculosis is associated with reduced sensitivity (14, 18), and although there have been substantial advances in scaling up, not all low income countries with high tuberculosis disease burden have the infrastructure to support high-level coverage (19).

3.2 WHO clinical screening tool for HIV-related pulmonary infection

To improve diagnosis of tuberculosis in high HIV prevalence settings, WHO introduced recommendations to guide initiation of empiric tuberculosis treatment, including a clinical screening algorithm for seriously ill patients presenting with cough of >2 weeks duration plus any of the following danger signs: respiratory rate >30 /min, fever >39^{o}C, pulse rate >120 /min or unable to walk unaided (20) (Appendix 1).
In response to the highlighting of weaknesses in the algorithm, WHO introduced a revised version in 2016 that included improvements such as defining the target patient as presenting with current cough instead of cough > 2 weeks (21) (Appendix 2). There are still obvious gaps. The algorithm recommends initiation of broad-spectrum antibiotics, and that treatment for PJP should be considered (without giving guidance on selection of patients for empiric PJP therapy). Diagnosis of tuberculosis is based on a rapid nucleic acid amplification test (the Xpert MTB/RIF assay), and empiric therapy for tuberculosis is recommended if the Xpert MTB/RIF assay is negative or unavailable and there is no response to antibiotics.

In the absence of optimal diagnostic facilities and tools, prescriber reliance on stepwise empiric treatment can delay commencing correct treatment, which adversely impacts morbidity and mortality

(22). On the other hand, inappropriate use of antimicrobials for patients whose diagnosis is unconfirmed poses risk of toxicity for the patient, incurs unnecessary costs, wastes valuable resources and results in increased resistance, threatening therapeutic and prophylactic antimicrobials (2, 10, 23). There is a pressing need for alternative diagnostic tests, suited to the triage of patients in high HIV burden, resource-constrained settings.

Procalcitonin (PCT) and C-reactive protein (CRP) are two non-specific inflammatory biomarkers that have been proposed as possible adjunct tests to diagnostic algorithms and treatment guidelines, to improve triage of patients and optimise the use of resources.

4. C-reactive protein and procalcitonin testing

4.1 C-reactive protein testing in non-HIV infected populations

C-reactive protein is a non-specific acute phase protein released by the liver in response to inflammatory cytokines (24) and used as a non-specific marker of inflammation. Typically, significantly higher serum concentrations are found in bacterial compared to viral infection and C-reactive protein testing has an established role in differentiating between the two (25). In a Korean study of non HIV-infected individuals in an intermediate tuberculosis burden setting, C-reactive protein has shown utility for distinguishing between CAP and tuberculosis (26).

4.2 CRP testing in HIV-infected patients

The findings of a 1993 Danish study suggested that CRP response is poorer in those with advanced HIV infection (27); however, a more recent study showed no correlation between C-reactive protein concentration and HIV status, degree of immunodeficiency or antiretroviral therapy (28). Elevated concentrations have been found in patients with CAP (7, 29, 30), whereas low to modest concentrations have been reported in patients with PJP (29-31). Active *Mycobacterium tuberculosis* infection in HIV-infected patients is associated with mild or moderate elevations in C-reactive protein

concentrations (7, 28, 30, 32, 33), and positive correlation between C-reactive protein concentrations and increased mycobacterial load, as well as increased concentrations in extra-pulmonary tuberculosis compared to pulmonary tuberculosis have been reported (17, 33).

Several South African studies have focused on the diagnostic value of C-reactive protein in ruling out TB in HIV-infected patients with smear negative disease (14, 33). Alvarez and colleagues demonstrated that C-reactive protein testing significantly improved the performance of a prediction model in diagnosing smear-negative tuberculosis in HIV-infected and -uninfected patients with suspected tuberculosis. Patients suspected as having tuberculosis with a normal C-reactive protein result were reclassified as low risk and allocated a follow-up visit, saving valuable resources and avoiding exposing patients to unnecessary investigations (8). Conclusions of a 2017 systematic review were that C-reactive protein was a promising tool for systematic screening for tuberculosis in HIV-infected adult populations (34). Availability of rapid, reliable, and inexpensive point-of-care tests would have obvious benefits in terms of facilitating earlier diagnosis, regardless of laboratory resources.

4.3 Point of care (POC) C-reactive protein (CRP)

POC CRP tests are widely used with a number of rapid, quantitative and semi-quantitative assays on the market. In 2014 a Cochrane Review concluded utility of POC C-reactive protein for guiding antibiotic therapy in patients with acute respiratory infections in primary health care settings (24), and the assay has been incorporated into management guidelines for suspected lower respiratory tract infection in high-income countries such as the UK (35).

POC CRP testing is suited to low-resourced settings in terms of portability and convenience and has shown utility in improving the management of tuberculosis with associated cost savings. In a South African study of HIV-infected smear-negative outpatients with suspected tuberculosis, Drain et al. demonstrated good sensitivity and moderate specificity of POC CRP combined with clinical criteria for improving diagnostic exclusion of tuberculosis (14). A Ugandan longitudinal study showed improved identification of HIV-infected participants requiring isoniazid preventive therapy for

tuberculosis when comparing the WHO symptom-screening algorithm with the addition of POC CRP testing against without POC CRP testing (36). The authors found a significant increase in the identification of those eligible for isoniazid preventive therapy and an associated decrease in referral for costly and time-consuming tuberculosis tests. In a 2017 study, Yoon et al. found 89% sensitivity and 72% specificity for POC CRP in active screening for tuberculosis in ART-naïve, HIV-infected adults. (37).

4.4 PCT testing in non-HIV infected populations

Procalcitonin is a calcitonin precursor, stimulated by inflammatory cytokines and bacterial endotoxins from parenchymal tissues and an established early marker of bacterial infection (24, 25). In healthy controls, procalcitonin is undetectable. Concentrations are typically high in bacterial infection whilst normal or mildly elevated procalcitonin associated with viral infection is due to the inhibition of its release by viral stimulation of interferon-gamma release (38, 39). Several studies have shown utility for procalcitonin for differentiating between systemic bacterial infection and viral infection in both immunocompetent and immunocompromised patients (25, 38, 40, 41), and between bacterial infection and systemic fungal infection (42). To date, the main utility of PCT testing for respiratory infections in non-HIV infected populations has been to rule-out bacterial infection, to aid appropriate antibiotic prescribing, and to predict severity of pneumonia and sepsis. Two Swiss randomized intervention trials of procalctionin-guided antibiotic use showed a 50% reduction in antibiotic exposure for patients presenting to emergency departments with lower respiratory infections (11), and a marked reduction in antibiotic prescription and duration in patients admitted with suspected CAP (12). A 2011 systematic review concluded that the use of procalcitonin to guide antibiotic therapy for respiratory infections was a safe and effective measure for reducing exposure to antibiotic therapy at all levels of care. (43). In contrast to bacterial infection, normal and moderately elevated procalcitonin concentrations have been observed in tuberculosis in non HIV-infected patients (26, 44).

4.5 PCT testing in HIV-infected patients

Comparing studies of procalcitonin concentrations in HIV-infected and non-HIV infected individuals, a meta-analysis by Huang et al. (2014) noted higher procalcitonin concentrations in those with tuberculosis and HIV infection than in those with tuberculosis and without HIV (45). HIV-infected individuals with tuberculosis have been found to have moderately elevated concentrations (7, 46) compared to higher concentrations in CAP with HIV co-infection, and highest concentrations found in pneumococcal pneumonia (6, 7). In a study of immunocompromised patients in intensive care units (including 31/119 infection), procalcitonin was found to accurately rule-out bacterial infection at a cut-off of 0.5 ng/ml (41).

4.6 POC procalcitonin testing

POC procalcitonin testing is a relatively new development. The miniVIDAS B.R.A.H.M.S PCT® assay was a rapid, automated assay and forerunner of POC procalcitonin testing. Evaluation of the laboratory-based assay in a validation study found good accuracy and correlation with reference standards as well as utility in early diagnosis of bacterial sepsis (40). In 2016, the POC enzyme-linked assay system miniVIDAS® was utilized in a treatment algorithm in a study that demonstrated reduced antibiotic use in patients with exacerbation of chronic obstructive pulmonary disease (47).

5. Literature review: utility of C-reactive protein and procalcitonin for differentiating between the tuberculosis, CAP and PJP in HIV-infected adults

Whilst the utility of the two biomarkers for ruling-out tuberculosis and CAP has been described by a number of studies, our specific interest is in the utility of the two biomarkers for seriously ill hospitalised HIV-infected adults presenting with symptoms of respiratory infection of unknown aetiology. We conducted a broad systematic literature search for studies involving HIV-infected individuals with one or more of the target infections and C-reactive protein and/or procalcitonin testing.

The following databases were searched using title/abstract and no limitations of dates, type of study or site of study: MEDLINE, Cochrane, Scopus, Web of Science and Africawide through EBSCO. Using Boolean strategy and Mesh terms, terms searched were: (1) subjects (“HIV” OR “human immunodeficiency virus” OR “AIDS” OR “Acquired Immunodeficiency Syndrome”); (2) disease (“opportunistic infections” OR "AIDS-Related Opportunistic Infections" OR “TB” OR “Mycobacterium” OR “MTB” OR “pneumocystis” OR “pjp” OR “pcp” OR “CAP” OR “pneumonia” OR “community acquired pneumonia” OR “Pneumonia, Pneumocystis" OR "Pneumocystis" OR "Pneumocystis jirovecii" OR "Pneumocystis carinii" OR "Pneumocystis Infections" OR "Tuberculosis" OR "Lung Diseases") and (3) tests (“PCT” OR “procalcitonin” OR “CRP” OR "C-Reactive Protein"). Forward checking was conducted along with crosschecking of bibliographies of relevant articles.

This search yielded few studies, particularly comparing all three target infections; fewer still were conducted in high prevalence HIV and TB settings. Disparity in findings may be attributable to variability in populations, assays and research methodology. Relevant studies reporting C-reactive protein and/or procalcitonin concentrations or diagnostic measures are summarised in table 1.

Table 1: Summary of studies reporting C-reactive protein and/or procalcitonin concentrations and/or diagnostic measures for tuberculosis (TB), bacterial community-acquired pneumonia (CAP) and *Pneumocystis jirovecii* pneumonia (PJP) in HIV-infected patients.

Study Authors (date)	Location (no. of sites)	Study design/ Participant selection (n)	Level of care	C-reactive protein (CRP) mg/mL, median (IQR)			Procalcitonin (PCT) ng/mL, median (IQR)			Diagnostic accuracy			
				TB	CAP	PJP	TB	CAP	PJP	Cut-off	Sens	Spec	Notes
High-income countries													
Grutzmeier/ Sandstrom (1999)	Sweden (1)	Prospective, febrile episodes (21)	Out-patients	-	115.5 (range 85-456)	12 (range 4-40)	-	-	-	-	-	-	
Benito et al. (2004)	Spain (1)	Prospective, new radiologic PIs (199)	In-patients	51 (69) myco-bacterial infection	102 (117)	38 (86)	-	-	-	>100	69%	83%	CAP vs. others/ combined with IL-8
Sage et al. (2010)	UK (1)	Case notes review, acute respiratory episodes (244)	In-patients	44 (range: <5-256)	120 (range: <5-620)	35 (range: <5-254)	-	-	-	-	-	-	Reported poor spec. Not provided
Cilloniz et al. (2013)	Spain (1)	Prospective, suspected CAP (155)	In-patients	-	-	-	-	-	-	≥220	35%	87%	Predicting CAP
										≥120	74%	53%	Predicting PJP
Lower-middle Income Countries													
Schleicher et al. (2005)	South Africa (1)	Prospective suspected CAP (67)	In-patients	177 (101)	341 (153.5)	-	1.03 (3.16)	19.05 (43.31)	-	>246	79%	82%	CRP
										>3	82%	82%	PCT
Nyamande et al. (2006)	South Africa (1)	Prospective suspected CAP (266)	In-patients	-	-	-	Mean-4.16 (SEM-1.20)	Mean-19.50 (SEM-5.64)	Mean-1.14 (SEM-0.29)				

Abbreviations: Sens, sensitivity; spec, specificity; PI, pulmonary infiltrate.

5.1 Diagnostic utility of procalcitonin

We identified two studies that evaluated procalcitonin in all three target infection. One South African study by Nyamande et al. compared procalcitonin concentrations in HIV-infected patients and found statistically significant differences in procalcitonin concentrations between the three target infections; however, no diagnostic accuracy measures were reported (6). The second by Schleicher et al. (also South African), involved bacterial pneumonia and tuberculosis, and demonstrated 82% sensitivity and 82% specificity at a cutoff value of 3 ng/ml. for discriminating pneumococcal pneumonia from tuberculosis (7). Both studies based participant selection on suspected CAP, and tuberculosis may have been underrepresented in analyses.

5.2 Diagnostic utility of C-reactive protein

Several studies report comparative C-reactive protein concentrations in HIV-infected patients with respiratory infections; although ranges differ widely, there is a general consensus that the highest concentrations are found in CAP compared to tuberculosis or PJP (7, 29, 30, 48). Conversely, a 1999 Danish study (27) failed to demonstrate a significant difference in C-reactive protein concentrations between CAP and PJP using a cut-off value of ≥80 mg/L.

We identified only two studies reporting diagnostic accuracy measures in all three of the target infections, with conflicting findings. A British case notes review study of HIV-infected adults admitted with respiratory infections found poor specificity in C-reactive protein for discriminating between the three infections (48), whereas a Spanish study found higher specificity (83%), and sensitivity 69%, for combined CRP and IL-8, in discriminating bacterial pneumonia from PJP or mycobacterial infections. The cohort included hospital-acquired infections and other mycobacterial infections beside tuberculosis (49).

A further two studies looked at two out of three of the target infections (7, 29). The first study by Cilloniz et al. involved 331 HIV-infected adults hospitalised with suspected CAP who were diagnosed with the following: bacterial and viral pneumonias, PJP, mixed infection, and unknown aetiology. The authors reported 35% sensitivity, 87% specificity at a cutoff value of 220 mg/L for predicting CAP,

and 74% sensitivity, 53% specificity at a cutoff value of 120 mg/L for predicting PJP (29). The other study by Schleicher (as for procalcitonin above) found sensitivity 79% and specificity 82% at a cutoff value of 246 mg/L in discriminating pneumococcal CAP from tuberculosis (7). Participant selection for both studies was based on patients presenting with suspected CAP.

5.3 Challenges to evaluation of biomarker diagnostic utility

Infection with more than one respiratory pathogen routinely presents a challenge to diagnostic research; dual or co-morbid infection is not uncommon. Cilloniz et al. found concomitant CAP and PJP infection in 17% (38/227) of participants (29). Nyamande et al. identified mixed infection in 21% (36/169) of participants in whom aetiology was determined, with higher mean procalcitonin concentrations in patients with tuberculosis/bacterial co-infection than in those with tuberculosis alone (6).

Disparity in procalcitonin response to different bacterial organisms may also limit ability of procalcitonin to differentiate between respiratory CAP and PJP. Nayamande found overlapping ranges between patients with atypical bacterial pneumonia and patients with PJP, consistent with a number of studies that report lower PCT concentrations in patients with atypical or Gram-negative infections (6, 12, 50). Furthermore, because patients are often commenced on antibiotics before attending higher facilities, changes in C-reactive protein concentrations in response to antibiotic therapy may impact findings (30).

6. Conclusion

Diagnosing and discriminating between respiratory infections of different aetiologies in the setting of HIV remains a challenge. Improved diagnostic algorithms using assays that are suited to low-resource settings would contribute to reducing delay to diagnosis and initiation of appropriate treatment. Although C-reactive protein and procalcitonin have shown some promise for discriminating between different respiratory infections in HIV-infected individuals, their performance is incompletely defined

and further studies are needed to evaluate their potential. Our overall hypothesis is that C-reactive protein and/or procalcitonin may have discriminative value as part of an improved algorithm in the triage of HIV-infected inpatients with respiratory infections; this study is designed to explore diagnostic utility of each biomarker as an initial step. For this purpose, we propose a secondary analysis of a large prospective HIV-infected cohort, recruited from two secondary level hospitals in Cape Town South Africa, serving communities with high HIV and TB prevalence. Patient enrollment was based on the 2007 WHO algorithm for seriously ill inpatients with suspected tuberculosis, but expanded to include cough of any duration in light of previous study findings, and reflects the population for whom the two biomarkers are being evaluated.

7. References

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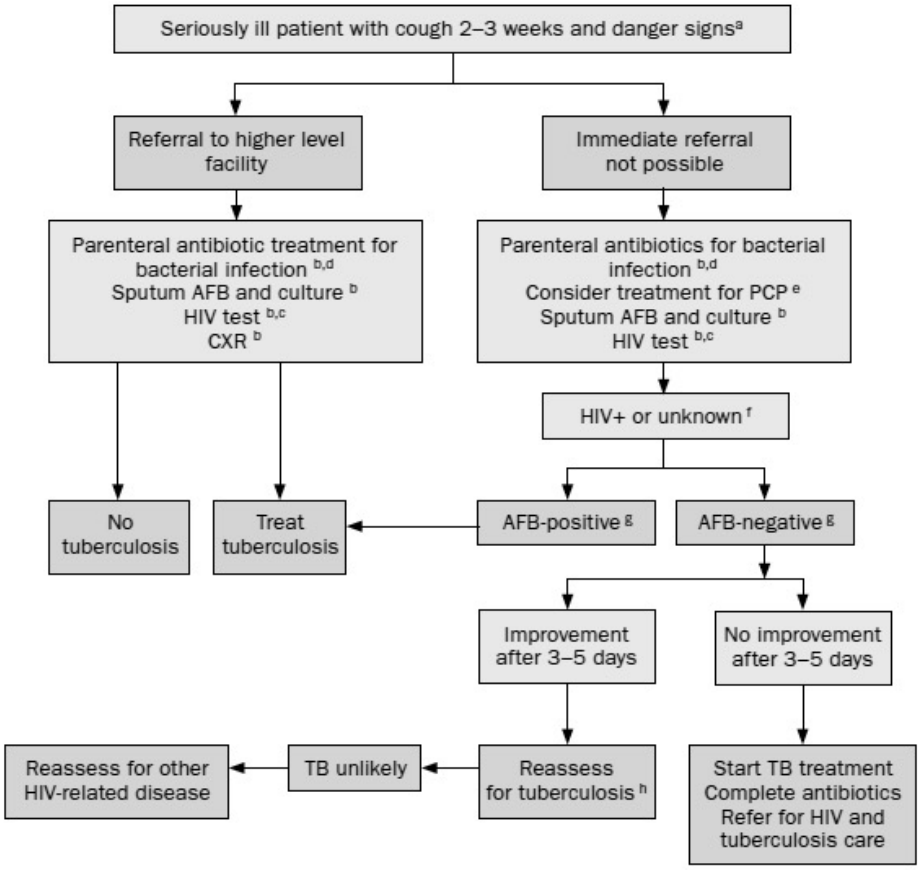
Appendix 1: 2007 WHO algorithm for the diagnosis of tuberculosis in seriously ill patients.

FIGURE 2

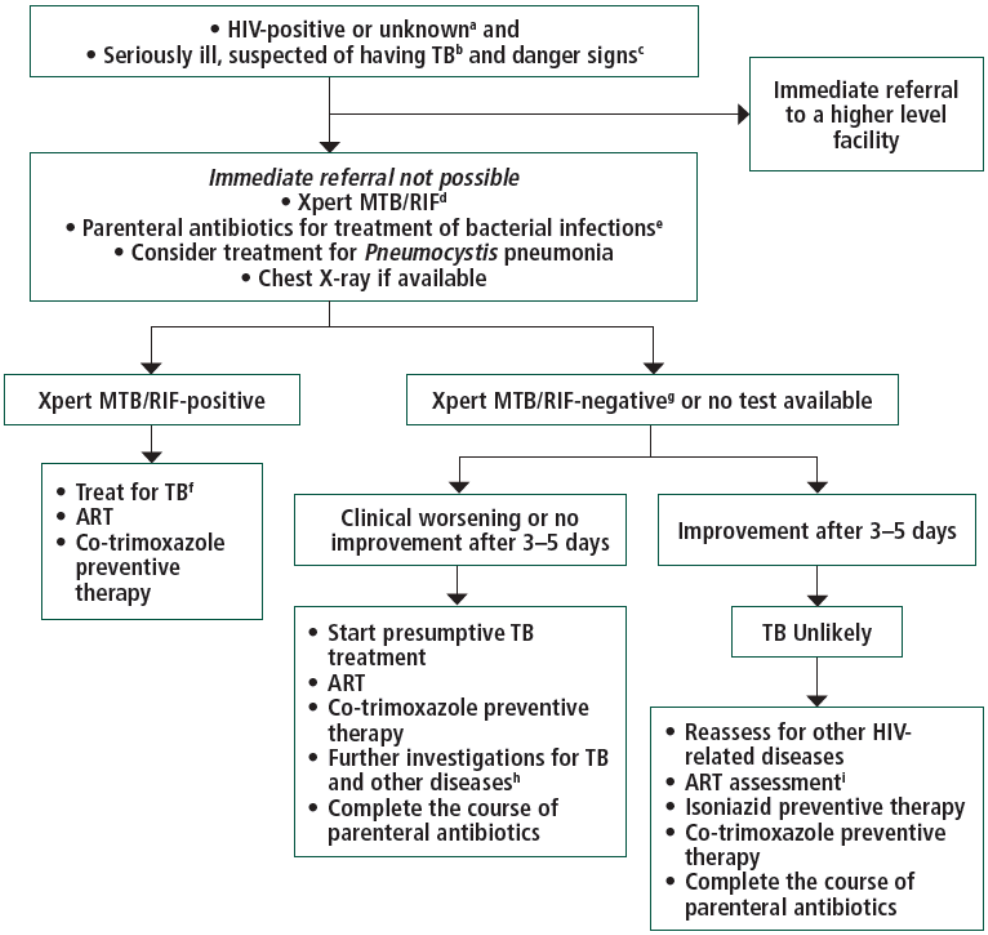
Algorithm for the diagnosis of tuberculosis in seriously ill patient in HIV-prevalent settings



[a] The danger signs include any one of: respiratory rate >30/min, fever >39 °C, pulse rate >120/min and unable to walk unaided.
[b] The investigations within the box should be done at the same time wherever possible in order to decrease the number of visits and speed up the diagnosis.
[c] For countries with adult HIV prevalence rate ≥1% or prevalence rate of HIV among tuberculosis patients ≥5%.
[d] Antibiotics (except fluoroquinolones) to cover both typical and atypical bacteria should be considered.
[e] PCP: *Pneumocystis carinii* pneumonia, also known as *Pneumocystis jirovecii* pneumonia.
[f] In the absence of HIV testing, classify HIV status unknown into HIV-positive depends on clinical assessment or national and/or local policy.
[g] AFB-positive is defined as at least one positive and AFB-negative as two or more negative smears.
[h] Reassessment for tuberculosis includes AFB examination and clinical assessment.

Appendix 2: 2016 WHO algorithm for managing people living with HIV and suspected of having TB (seriously ill).

Algorithm for managing people living with HIV and suspected of having TB (seriously ill)



[a] For all people with unknown HIV status, HIV testing should be performed according to national guidelines.

[b] Suspicion of TB is defined by the presence of any one of the following symptoms.
- For adults and adolescents living with HIV: current cough, fever, weight loss or night sweats.
- For children living with HIV: poor weight gain, fever, current cough or history of contact with a TB case.

[c] Danger signs include any one of the following: respiratory rate>30 per minute, temperature >39°C, heart rate >120 beats per minute and unable to walk unaided.

[d] For people suspected of having extrapulmonary TB, extrapulmonary specimens shoud be obtained for Xpert MTB/RIF (cerebrospinal fluid, lymph nodes and other tissues: Xpert MTB/RIF has low sensitivity for pleural fluid and data are limited for stool, urine or blood). The urine lateral flow lipoarabinomannan (LF-LAM) assay may be used to assist in diagnosing active TB among seriously ill adults and children living with HIV, regardless of CD4 count.

[e] Antibiotics with broad-spectrum antibacterial activity (except fluoroquinolones) should be used.

[f] If Xpert MTB/RIF shpws rifampicin resistance, treatment for multidrug-resistant TB should be initiated. If the person is considered at low risk for rifampicin resistance, a second Xpert MYB/RIF test should be performed on a fresh specimen. Collect and refer a sample for culture and additional drug sensitivity testing.

[g] If Xpert MTB/RIF shows negative results, the test can be repeated using a fresh specimen.

[h] Further investigations for TB include chest x-ray, clinical assessment, a repeat Xpert MTB/RIF on a fresh specimen and culture. If extrapulmonary TB is suspected, extrapulmonary specimens should be obtained and sent for culture and abdominal ultrasound may be performed.

[i] ART should be recommended for adults, regardless of CD4 count or clinical stage.

Student: Fiona Mendelson (MNDFIO001), 19th Feb 2018

PART C: Manuscript

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Diagnostic accuracy of C-reactive protein and procalcitonin to discriminate between tuberculosis, *Pneumocystis jirovecii* pneumonia, and bacterial pneumonia in HIV-infected inpatients meeting WHO criteria for seriously ill.

Short title: Diagnostic accuracy of C-reactive protein and procalcitonin and respiratory infections in HIV

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Short title: Diagnostic accuracy of C-reactive protein and procalcitonin and respiratory infections in HIV

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Abstract

Tuberculosis, bacterial community-acquired pneumonia (CAP), and *Pneumocystis jirovecii* pneumonia (PJP) are major causes of hospitalisation in HIV-infected individuals. Prompt diagnosis and treatment initiation are important to reduce morbidity and mortality, but are hampered by limited diagnostic resources in resource-poor settings. C-reactive protein (CRP) and procalcitonin have shown diagnostic utility for respiratory tract infections, however few studies have focussed on their ability to distinguish between tuberculosis, CAP, and PJP in HIV-infected inpatients.

We evaluated the diagnostic accuracy of CRP and procalcitonin, compared with composite reference standards, to discriminate between the three target infections in adult HIV-infected inpatients in two district level hospitals in Cape Town, South Africa, admitted with current cough and danger signs in accordance with the WHO algorithm for tuberculosis in seriously ill HIV-infected patients. Study clinicians were blinded to CRP and procalcitonin results.

248 participants met study case definitions: 133 with tuberculosis, 61 with CAP, 16 with PJP, and 38 with mixed infection. The differences in median CRP and procalcitonin concentrations between the three infections were statistically significant, but distributions overlapped considerably. CRP and procalcitonin cut-offs with sensitivities of ≥ 90% were found for all three target infection pairs, however corresponding specificities were low. Receiver operating characteristic areas under the curve for CRP and procalcitonin were between 0.68 and 0.74 for PJP versus tuberculosis and PJP versus CAP.

CRP and procalcitonin showed limited value in discriminating between the three target infections due to widely overlapping distributions, but diagnostic accuracy was higher for discriminating PJP from CAP or tuberculosis. Our findings suggest that CRP and procalcitonin may have greater diagnostic utility as part of a panel of biomarkers or in clinical prediction rules.

Introduction

Respiratory infections are a major cause for hospital admission in HIV-infected people globally in the antiretroviral era; the commonest being tuberculosis, bacterial community-acquired pneumonia (CAP), and *Pneumocystis jirovecii* pneumonia (PJP) (1). Prompt diagnosis and initiation of appropriate treatment is important to reduce mortality in HIV-infected inpatients.

Determining the aetiology of serious infections in inpatients with HIV is challenging, in part due to considerable overlap in the clinical and radiographic presentation of tuberculosis, CAP, and PJP (2). Atypical presentation and dual infection further compounds these diagnostic challenges. Furthermore, there are limitations of current diagnostic methods and limited access to diagnostic tests in resource poor settings (3, 4, 5).

WHO's algorithm for the diagnosis of tuberculosis in seriously ill patients (6) recommends broad spectrum antibiotics, that treatment for PJP should be considered (without giving guidance on selection of patients for empiric PJP therapy), using a rapid nucleic acid amplification test (the Xpert MTB/RIF assay) to diagnose tuberculosis, and empiric therapy for tuberculosis if the Xpert MTB/RIF assay is negative or unavailable and there is no response to antibiotics. Our group recently reported that 91.5% of patients defined as seriously ill by WHO and who had a current cough were diagnosed with tuberculosis, CAP, and/or PJP (7). Simple affordable tests to discriminate between these three infections could improve outcomes in seriously ill patients. Previous studies have proposed the use of biomarkers of inflammation as triage tests in clinical algorithms. Two of these, C-reactive protein (CRP) and procalcitonin, have shown some diagnostic utility for bacterial respiratory infections (8, 9). An additional advantage is that both CRP and procalcitonin are available as affordable point-of-care tests (10, 11). C-reactive protein is a non-specific acute phase protein released by the liver in response to inflammatory cytokines (12). Typically, significantly higher serum concentrations are found in bacterial compared to viral infection (13), and low to modest concentrations have been reported in patients with PJP (14). CRP has shown high sensitivity but low specificity for diagnosing HIV-associated tuberculosis (8).

Procalcitonin is a calcitonin precursor, stimulated by inflammatory cytokines and bacterial endotoxins from parenchymal tissues and an established early marker of bacterial infection (12, 13).

Studies have shown utility for procalcitonin for differentiating between systemic bacterial infection and viral infection (13, 15), and between bacterial infection and systemic fungal infection (16). However, only two studies have examined diagnostic performance of CRP in discriminating between tuberculosis, CAP, and PJP in hospitalised patients with HIV, with conflicting results (17, 18). We were unable to find any studies reporting diagnostic accuracy of procalcitonin in discriminating between all three infections.

The purpose of this study was to explore the diagnostic accuracy of CRP and procalcitonin in predicting presence or absence of each of the three major infections in seriously ill inpatients with HIV infection. Secondary objectives were to describe the extent to which CRP and procalcitonin concentrations differed between the three infections and to determine optimal concentration cut-offs for discriminating those with and without each target infection.

Methods

Study Setting and Participants

We conducted a secondary analysis of a sub-set of prospective cohort data from a larger study (7), which was designed to improve the evidence base for the WHO algorithm for the diagnosis of tuberculosis in seriously ill HIV-infected participants with current cough (3). Recruitment for the main study took place at two secondary level hospitals in Cape Town, South Africa, serving communities with high HIV and tuberculosis prevalence: G.F. Jooste District Hospital from November 2011 until the hospital's closure in February 2013, and Khayalitsha District Hospital from March 2013 until October 2014.

Inclusion criteria for the main study were: admission into the enrollment facility within the previous 24 hours, ≥18 years of age, known HIV infection, cough of any duration, and at least one WHO-defined danger sign (respiratory rate >30/min, fever >39^{o}C, pulse rate >120/min, and unable to walk unaided). Exclusion criteria were: current, recent, or defaulted anti-tuberculosis treatment;

exacerbation of either congestive cardiac failure or chronic obstructive pulmonary disease; and failure to provide a spontaneous or induced sputum specimen.

For the current study we added the following two inclusion criteria: 1) participants fulfilling our case definitions for tuberculosis, CAP, and/or PJP, and 2) participants with both a CRP and procalcitonin test result (assays done in a batch at the end of the study).

Case definitions

1) Tuberculosis: positive *Mycobacterium tuberculosis* culture from any site plus at least one symptom consistent with tuberculosis (cough, fever, night sweats, weight loss).

2) CAP: cough ≤ 14 days plus one or more additional respiratory symptoms (sputum, breathlessness, chest pain, haemoptysis or fever) plus radiological evidence of pulmonary consolidation (confirmed by a radiologist) (19).

3) PJP: cough ≤ three months plus radiological evidence of diffuse bilateral interstitial infiltrates (confirmed by a radiologist) plus oxygen saturation ≤ 92% (adapted from Centers for Disease Control and Prevention case definition) (20).

Investigations

Three sputum specimens were obtained from each participant. One sample was sent for Gram stain, culture, and sensitivity, and two samples for smear examination with auramine staining for acid-fast bacilli (AFB) and liquid mycobacterial culture (BACTEC™ MGIT™ 960; Becton, Dickinson and Company, New Jersey, USA). Mycobaterial blood culture was done on all participants. Mycobacterial culture was done on other specimens when appropriate (e.g. pleural fluid). Serum β-D-glucan assay (Fungitell™; Associates of Cape Cod, Inc., east Falmouth, MA, USA) was done on all participants.

CRP and procalcitonin tests were done on stored serum in a batch after the study, therefore these tests had no role in patient management. Laboratory staff were blinded to participant diagnosis and outcome. Assays used were: Siemens Advia 1800 for CRP and Siemens Advia Centaur XP for

procalcitonin. Assay range for CRP was 4-[304-336] mg/L, normal range was below 10 mg/L. Assay range for procalcitonin was <0.02-75 µg/L, normal range below 0.02 µg/L.

Chest radiographs were performed on admission and retrospectively reviewed by a senior radiologist blinded to diagnoses and results of laboratory investigations.

Statistical analyses

All analyses were performed using Stata software version 13.0 (StataCorp Inc, College Station, Texas, USA).

Based on our fixed sample size, we explored precision to detect 90% sensitivity for each index test for the three target infections, aiming for a maximum ±10% variation in 95% confidence intervals (CIs). We were unable to find data on sensitivity estimates for procalcitonin in all three target infections in HIV-infected individuals, therefore calculations were based exclusively on studies reporting CRP measures of diagnostic accuracy. We estimated a range of confidence intervals of binomial proportions using the Wilson-score interval for smaller sample sizes (21). Since our data was not normally distributed, a second calculation was made using 85% of the original sample sizes as suggested by Lehmann et. al (22). We estimated the 95% CIs of 90% sensitivity to be 83%-94% for tuberculosis, 79%-96% for CAP, and 69%-99% for PJP. The small sample size for PJP accounted for wide 95% confidence intervals. Further details of these sample size calculations are provided in table S1.

To detect differences in CRP concentrations between the three target infections, we estimated power for a two-sample means test (assuming unequal variances), based on relevant literature. (Expected means for CRP were approximate due to lack of reported standard deviations for tuberculosis or CAP). Our study had 80% power and alpha of 0.05 (using 85% of the original sample size to account for non-normal distribution of CRP and procalcitonin concentrations) to detect a minimum mean concentration difference in CRP between tuberculosis and PJP of 36%, between CAP and PJP of 14%, and between CAP and tuberculosis of 14%, and a minimum mean concentration difference in

procalcitonin of 50% between tuberculosis and PJP, 62% between CAP and PJP, and 62% between CAP and tuberculosis. Further details of these sample size calculations are provided in table S2.

Diagnostic accuracy analyses for CRP and procalcitonin were performed for participants fulfilling criteria for one of the three single infection definitions. Participants with mixed infection were analysed separately. In clinical practice, differential diagnostic challenges usually present between two of the target infections and less commonly between all three, hence we calculated diagnostic accuracy measures between infection pairs in addition to each target infection versus the other two.

As distributions of both CRP and procalcitonin were not normally distributed, we used non-parametric statistical tests for continuous variables. Univariate associations between participant baseline characteristics in infection pairs were analysed using the Wilcoxon-Mann-Whitney test for continuous data, and Chi-square test (or Fisher's Exact test if values in a cell were <5), for categorical data. All P tests were two-tailed.

For biomarker analyses, CRP and procalcitonin values below the detectable limit (BDL) of the assay were substituted with half BDL (in preference to substitution with the assay limit or with zero, both of which have been shown to bias parameter estimates) (23).

Receiver Operating Characteristic (ROC) area under the curve (AUC) analyses were used to explore potential cut-offs for CRP for each target infection using Liu's index (24), which we then used to calculate diagnostic accuracy estimates. Cut-offs were also explored using the WHO 90% sensitivity recommendation for screening tests for tuberculosis (25). To mitigate overfitting and improve accuracy of model prediction, we performed cross-validation on all ROC AUC's exceeding 60% using k-fold cross-validation, as the dataset was too small for generation of a training set.

Since there are few studies on the diagnostic accuracy of procalcitonin for infections in HIV-infected patients, we explored cut-offs established for both lower respiratory tract infections (LRTI) and sepsis. Procalcitonin categories for LRTI were: < 0.1 µg/L, bacterial infection very unlikely; 0.1 - 0.25 µg/L, localised bacterial infection unlikely; 0.25 - 0.5 µg/L, localised bacterial infection possible; >

0.5 µg/L, suggestive of bacterial infection. Procalcitonin categories for systemic bacterial infection / sepsis were: 0.5 – 2 µg/L, systemic infection possible; 2 – 10 µg/L, suggestive of systemic infection; > 10 µg/L, severe systemic infection / septic shock (9).

Ethics

The University of Cape Town human research ethics committee approved both main and current studies (HREC REF 334/2011, renewal dates: 30/05/2016 and 30/11/2018). Participants who met inclusion criteria were invited to participate in the study and written informed consent obtained. Participants who were admitted in a confused state were enrolled and offered the option of remaining in the study once orientated.

This study conforms to the Standards for Reporting Diagnostic Accuracy Studies (STARD) guidelines (26).

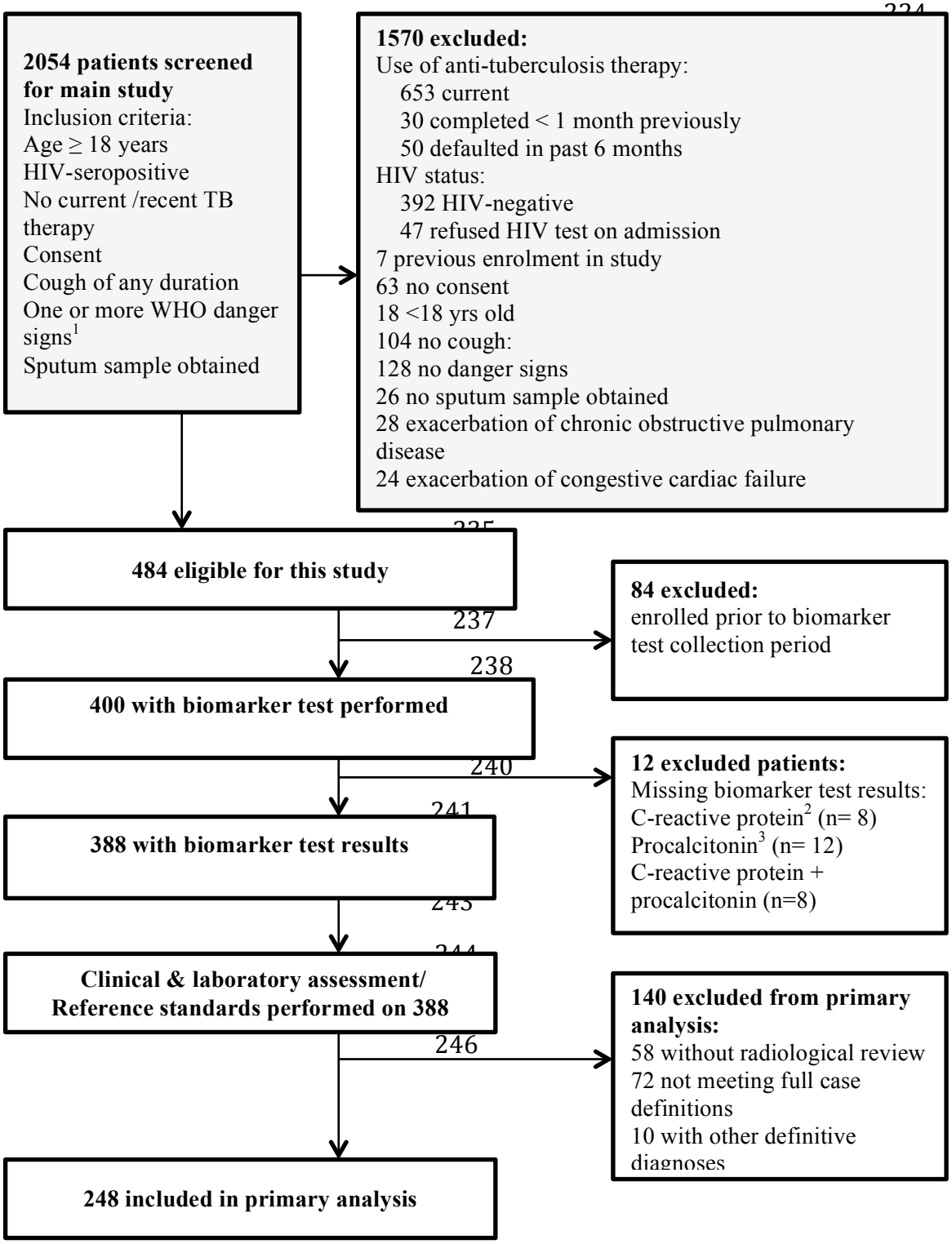
Results

Participant characteristics

500 participants were screened for this study. 4 participants without danger signs and 84 enrolled prior to biomarker testing were excluded from the current study. A further 12 participants with missing index test results and 58 participants without radiological reviews were also excluded. The other 72 participants received empirical diagnoses of one or more of the three target infections, but did not meet study case definitions. Ten participants were excluded due to diagnoses other than the three target infections (meningitis, disseminated *Cryptococcus,* emmonsia, bronchitis, and post-TB bronchiectasis with a pneumothorax).

The participant flow diagram is shown in Figure 1.

Figure 1. Consort diagram based on Standards for Reporting Diagnostic Accuracy Studies (STARD).



[1] WHO danger signs: respiratory rate >30 /min, fever >39^{o}C, pulse rate >120 /min or unable to walk unaided.

Figure 2 summarises distribution of target infections in 210 participants with one of the three target infections. All participants had received at least one dose of antibiotics use prior to obtaining blood samples for biomarker tests. 73/248 (29%) reported taking cotrimoxazole prophylaxis prior to admission. Univariate analysis of variables associated with each of the three single infection diagnoses are shown in table 1. Tuberculosis was statistically associated with lower haemoglobin concentrations and reported weight loss compared with the other two groups. Inability to walk unaided was more common in those with tuberculosis than in those with CAP. Those with CAP had statistically higher median white cell count and CD4 count than the other two infections. The CAP group was more likely to have been using antiretroviral therapy prior to admission compared with the tuberculosis group. Participants with PJP had statistically lower CD4 counts, and were more likely to have a respiratory rate above 30/minute than participants in the other two groups.

Figure 2. Number of participants diagnosed with single target infections and mixed infections. Tuberculosis (PTB), bacterial community acquired pneumonia (CAP) and *Pneumocystis jirovecii* pneumonia (PJP), n (%). N = 248

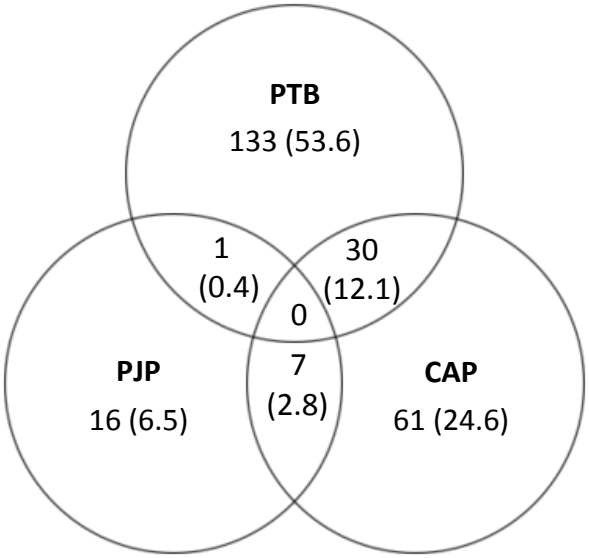


Table 1: Baseline characteristics of 210 participants with a single target infection by infection status

Diagnosis n (%)	Total N=210	TB n=133 (63)	CAP n=61 (29)	PJP n=16 (8)	P-value for pairwise comparison*
Median age in yrs. (IQR)	34.8 (28.9-40.7)	34.7 (29.1-40.8)	35.1 (29.4-40.0)	36.9 (28.8-41.2)	TB vs. CAP: 0.96, CAP vs. PJP: 0.84 PJP vs. TB: 0.75
Sex: female n(%)	139 (66)	84 (63)	44(72)	11(69)	TB vs. CAP: 0.22, CAP vs. PJP: 0.71# PJP vs. TB: 0.79#
Cotrimoxazole prophylaxis	60 (29)	38(29)	18(30)	4(25)	TB vs. CAP: 0.89, CAP vs. PJP: 0.49# PJP vs. TB: 1.00#
Antiretroviral therapy n (%)	76 (36)	43(32)	29(48)	4(25)	TB vs. CAP: 0.04, CAP vs. PJP: 0.16# PJP vs. TB: 0.78#
Median $CD4^+$ count, cells/µL (IQR)	97 (38-210)	77 (35-162)	200 (79-287)	35 (12-81)	TB vs. CAP: 0.0001 CAP vs. PJP: 0.0005, PJP vs. TB: 0.03
Median WCC $x10^9$/L (IQR)	8.6 (5.8-12.9)	7.3 (5.2-10.2)	12.3 (8.4-20.0)	8.2 (6.2-10.7)	TB vs. CAP: 0.0001, CAP vs. PJP: 0.01 PJP vs. TB: 0.5
Median Hb g/dL (IQR)	9.4 (7.7-10.8)	8.6 (7.4-10.1)	10.4 (8.8-12)	11.25 (9.7-12.2)	TB vs. CAP: 0.0001, CAP vs. PJP: 0.24 PJP vs. TB: 0.0001
β-D-glucan > 300 pg/mL	25 (12)	11 (8)	1 (2)	13 (80)	TB vs. CAP: 0.11#, CAP vs. PJP: <0.0001# PJP vs. TB: <0.0001#
WHO danger signs[1]:					
Pulse rate> 120beats/min	166 (79)	106 (80)	51 (84)	9 (56)	TB vs. CAP: 0.52 , CAP vs. PJP: 0.02 PJP vs. TB: 0.04
Respiratory rate>30/min	137(65)	83 (62)	38 (62)	16 (100)	TB vs. CAP: 0.99 , CAP vs. PJP: 0.002# PJP vs. TB: 0.001#
Fever>39°C	31 (15)	20 (15)	10 (16)	1 (6)	TB vs. CAP: 0.81, CAP vs. PJP: 0.44# PJP vs. TB: 0.47#
Unable to walk unaided	119 (57)	88 (67)	23 (38)	8 (50)	TB vs. CAP:<0.0001, CAP vs. PJP: 0.40 PJP vs. TB: 0.19
TB symptoms[2]:					
Night sweats	137 (66)	89 (67)	38 (63)	10 (67)	TB vs. CAP: 0.58 , CAP vs. PJP:1.00# PJP vs. TB: 0.78#
Weight loss	196 (94)	130 (98)	53 (88)	13 (81)	TB vs. CAP: 0.005# , CAP vs. PJP: 0.43# PJP vs. TB: 0.009#
Fever	170 (82)	106 (80)	52 (87)	12 (75)	TB vs. CAP: 0.29, CAP vs. PJP: 0.27# PJP vs. TB: 0.74#

Abbreviations: TB, tuberculosis; CAP, bacterial community-acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia; Hb: haemoglobin; WCC: white cell count

*Hypothesis tests- Wilcoxon-Mann-Whitney test for continuous data; Chi-square test for categorical data. #Fisher's exact test where 1 or more cells <5

[1] Danger signs based on WHO algorithm for diagnosis of TB in seriously ill patients; [2]Cough of any duration was a study inclusion criterion

CRP concentrations and diagnostic utility for each infection

Distributions of CRP concentrations by single infection category are shown in figure 3A. Comparison of CRP concentrations between the three single infections are shown in table 2. Elevated concentrations (>10mg/L) were found in 206/210 (98%) participants: 131/133 (98%) with tuberculosis, 60/61 (99%) with CAP, and 15/16 (94%) with PJP. There were statistically significant differences in median concentrations between infection pairs, with considerable overlap in distributions. The highest concentrations were in participants with CAP and the lowest concentrations in those with PJP.

Figure 3. Distribution of **(A)** C-Reactive Protein and **(B)** procalcitonin by diagnosis. Bacterial community acquired pneumonia (CAP), *Pneumocystis jirovecii* pneumonia (PJP).

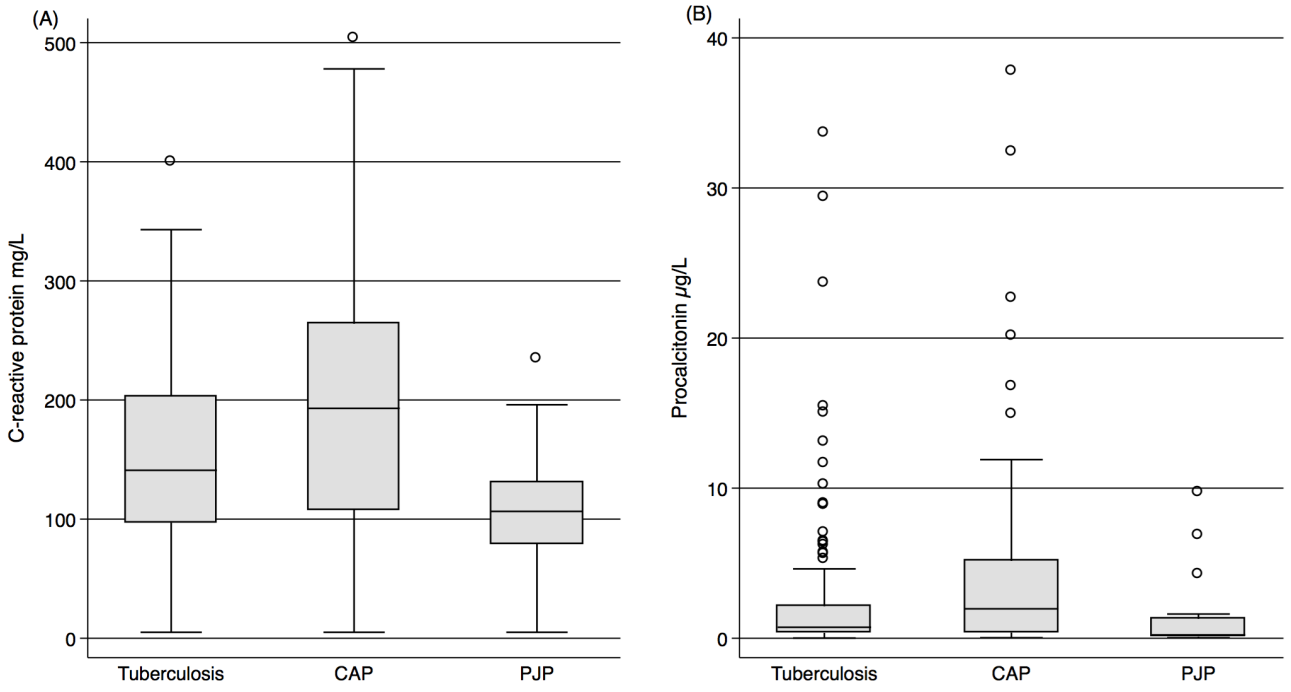


Table 2: C-reactive protein and procalcitonin distributions by infection in 210 participants with a single target infection

Biomarker concentration	Total (N=210)	TB (n=133)	CAP (n=61)	PJP (n=16)	P-value *
Median CRP mg/L (IQR)	148 (96-224)	141 (97-203)	193 (108-264)	106.5 (79.5-131.5)	TB vs.CAP: 0.02, CAP vs. PJP: 0.003 PJP vs. TB: 0.02
CRP ≥10mg/L[1] n (%)	206 (98)	131 (98.5)	60 (98.4)	15 (93.8)	TB vs.CAP: 1.00[#], CAP vs. PJP: 0.38[#] PJP vs. TB: 0.29[#]
Median PCT µg/L (IQR)	0.8 (0.3-2.9)	0.7 (0.4-2.1)	2.0 (0.4-5.2)	0.2 (0.1-1.3)	TB vs. CAP: 0.05, CAP vs. PJP: 0.01 PJP vs. TB: 0.05
PCT ≥0.02 µg/L[2]: n (%)	209 (99.5))	132 (99)	61 (100)	16 (100)	-
PCT ≥0.1 µg/L: n (%)	199 (94.8)	128 (96.2)	58 (95.1)	13 (81.3)	TB vs. CAP: 0.71[#], CAP vs. PJP: 0.10[#] PJP vs. TB: 0.04[#]
PCT ≥0.25 µg/L: n (%)	170 (81)	112 (84)	50 (82)	8 (50)	TB vs. CAP: 0.70, CAP vs. PJP: 0.008 PJP vs: TB: 0.001
PCT ≥0.5 µg/L: n (%)	137 (65.2)	87 (65.4)	43 (70.5)	7(43.8)	TB vs. CAP: 0.49, CAP vs. PJP: 0.05 PJP vs. TB: 0.11
PCT ≥2 µg/L : n (%)	69 (32.9)	36 (27.1)	30 (49.2)	3 (18.8)	TB vs. CAP: 0.003, CAP vs. PJP: 0.05[#] PJP vs. TB: 0.56[#]
PCT >10 µg/L: n (%)	16 (8)	8 (6)	8 (13)	0 (0)	-

Abbreviations: CRP, C-reactive protein; PCT, Procalcitonin; TB, tuberculosis; CAP, bacterial community acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia
[1] Elevated concentration. [2]Lower detectable limit for procalcitonin assay.
[*]Hypothesis tests- Wilcoxon-Mann_Whitney test for non-normally distributed continuous data and Chi-square test for categorical data. [#]Fisher's exact test where 1 or more cells <5 .

ROC AUCs for CRP for each disease pair versus the other two are shown in figure 4. Cross-validation showed minor reductions of ROC AUCs, from 0.60 to 0.58 (95% CI : 0.49-0.67) in the tuberculosis versus CAP group; from 0.74 to 0.72 (95% CI: 0.59-0.84) for CAP versus PJP; and from 0.68 to 0.64 (95% CI: 0.51-0.77) for PJP versus tuberculosis. Diagnostic accuracy estimates for two cut-offs for each infection pair and each infection versus the other two infections are presented in table 3. CRP cut-offs with reasonable diagnostic accuracy were found for PJP versus CAP, PJP versus tuberculosis and PJP versus the other two infections.

Figure 4. ROC curves for C-reactive protein and procalcitonin for each diagnosis within infection pairs.
(A) Tuberculosis versus bacterial community acquired pneumonia, **(B)** community-acquired bacterial pneumonia versus *Pneumocystis jirovecii* pneumonia, and **(C)** *Pneumocystis jirovecii* pneumonia versus tuberculosis.
C-reactive protein (CRP), procalcitonin (PCT), and area under the receiver operating characteristic Curve (AUC).

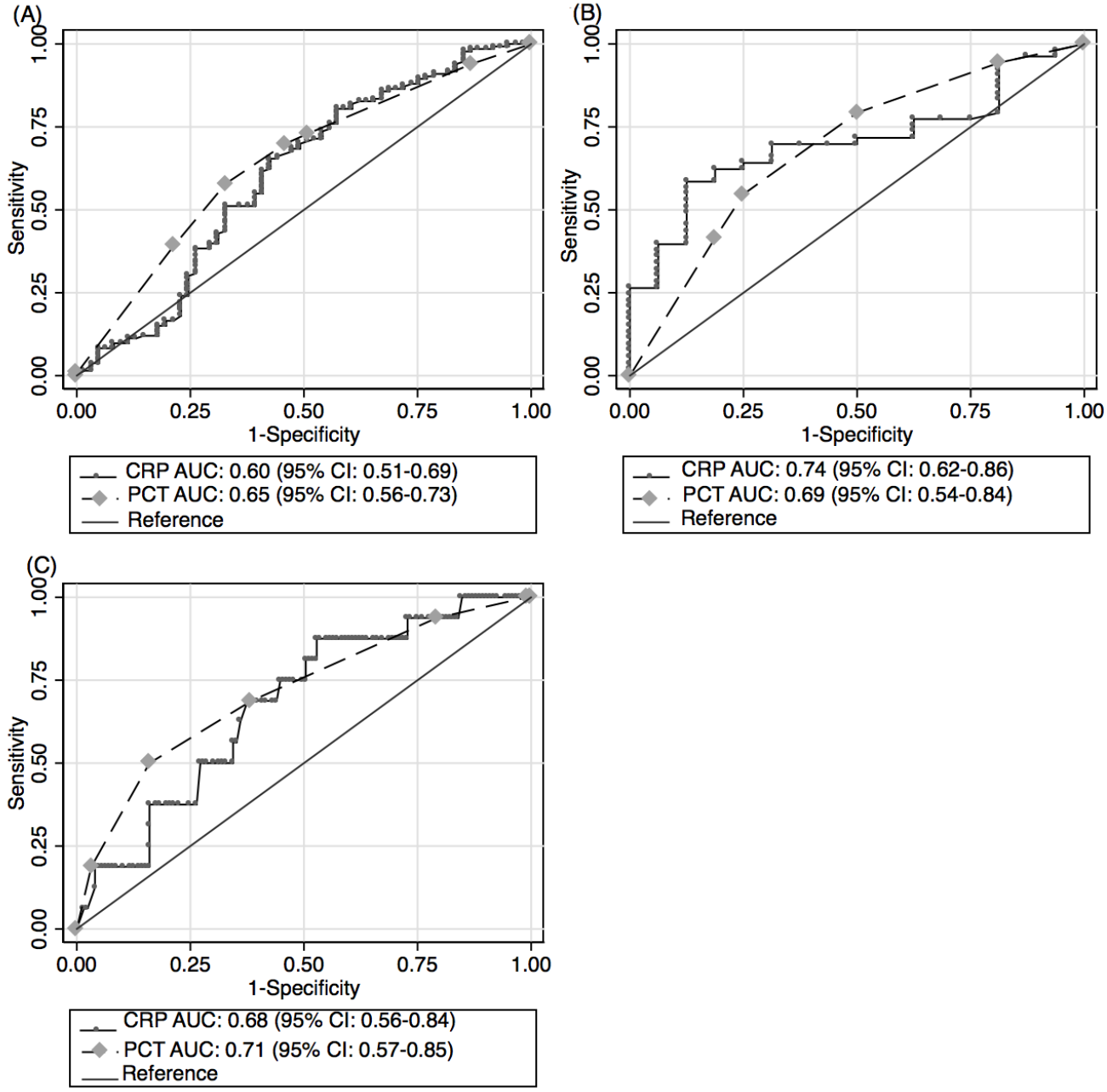


Table 3: Diagnostic accuracy of C-reactive protein for target infections.
Two cut-offs are listed for each infection pair and one infection versus the other two: the first with minimum 90% sensitivity and the second selected using the Liu index (see text for details).

Infection*	Cut-off (mg/L)	Sensitivity% (95% CI)	Specificity% (95% CI)	LR+ (95% CI)	LR- (95% CI)	Diagnostic odds ratio (95% CI)
TB vs. CAP	CRP ≥64	90.2 (83.9-94.7)	11.5 (4.7-22.2)	1.02 (0.92-1.13)	0.85 (0.36-2.03)	1.20 (0.45-3.17)
	CRP <175	65.4 (56.7-73.4)	57.4 (44.1-70.0)	1.53 (1.12-2.11)	0.60 (0.44 -0.83)	2.55 (1.37-4.72)
CAP vs. PJP	CRP ≥ 63	90.2 (79.8- 96.3)	18.8 (4.0-45.6)	1.11 (0.86-1.42)	0.52 (0.15-1.87)	2.12 (0.51-8.94)
	CRP ≥ 147	63.9 (50.6-75.8)	87.5 (61.7-98.4)	5.11 (1.38-18.96)	0.41 (0.28-0.60)	12.41 (2.83-59.7)
PJP vs. TB	CRP ≥33	93.8 (69.8-99.8)	2.3 (0.5-6.5)	0.96 (0.84-1.09)	2.77 (0.31-25.08)	0.35 (0.03-3.54)
	CRP <147	87.5 (61.7-98.4)	48.9 (40.1-57.7)	1.71 (1.33-2.19)	0.26 (0.07-0.95)	6.69 (1.46-30.60)
TB vs. CAP/PJP	CRP ≥64	90.2 (83.9-94.7)	13.0 (6.4-22.6)	1.04 (0.94-1.15)	0.75 (0.35-1.63)	1.38 (0.58-3.25)
	CRP ≥150	48.9 (40.1-57.7)	49.4 (37.8-61.0)	0.96 (0.73-1.28)	1.04 (0.78-1.37)	0.93 (0.53-1.63)
CAP vs. TB/PJP	CRP ≥63	90.2 (79.8-96.3)	10.7 (6.3-16.9)	1.01 (0.91-1.12)	0.92 (0.38-2.23)	1.10 (0.42-2.87)
	CRP ≥175	57.4 (44.1-70.0)	67.8 (59.6-75.2)	1.78 (1.30-2.45)	0.63 (0.46-0.86)	2.83 (1.54-5.21)
PJP vs. TB/CAP	CRP ≥33	93.8 (69.8-99.8)	2.6 (0.8-5.9)	0.96 (0.85-1.09)	2.42 (0.30-19.52)	0.40 (0.04-3.62)
	CRP <147	87.5 (61.7-98.4)	53.6 (46.3-60.8)	1.89 (1.48-2.40)	0.23 (0.06-0.86)	8.09 (1.99- 36.55)

Abbreviations: TB, tuberculosis; CAP, bacterial community acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia; PPV, positive predictive value; NPV, negative predictive value; LR, Likelihood ratio; CI, confidence interval. *Cohort prevalences: TB, 63,3% (95%CI, 56.4%-69.9%); CAP, 29.0% (95% CI, 23.0%-35.7%); PJP, 7.6% (95% CI, 4.4%-12.1%)

Procalcitonin concentrations and diagnostic utility for each infection

Distributions of procalcitonin category in each of the three target infections are shown in figure 3B and table 2. We found highest procalcitonin concentrations in the CAP group and lowest in those with PJP. There were statistically significant differences in median procalcitonin concentrations between

the three infection groups, but there was marked overlap in distributions. Diagnostic performance of procalcitonin categories for discriminating between infection pairs is shown in table 4. ROC AUCs for procalcitonin for each disease pair versus the other two are shown in figure 4. Best performance of procalcitonin was in discriminating between CAP and PJP.

Table 4: Diagnostic accuracy of procalcitonin by category for target infection pairs. Selected categories are based on assay guidelines developed for antibiotic use guidance in lower respiratory tract infections and sepsis (see text for details).

Infection pair*	Cut-off (µg/L)	Sensitivity% (95% CI)	Specificity% (95% CI)	LR+	LR-	Diagnostic odds ratio (95% CI)
TB vs. CAP	**PCT ≥0.1**	96.2 (91.4-98.8)	4.9 (1.0-13.7)	1.01 (0.95-1.08)	0.76 (0.19-3.10)	1.32 (0.34-5.21)
	PCT ≥0.25	84.2 (76.9-90.0)	18.0 (9.4-30.0)	1.03 (0.89-1.18)	0.88 (0.45-1.70)	1.17 (0.53-2.59)
	PCT ≥0.5	65.4 (56.7-73.4)	29.5 (18.5-42.6)	0.93 (0.76-1.14)	1.17 (0.75-1.84)	0.79 (0.41-1.52)
	PCT ≥2	27.1 (19.7-35.5)	50.8 (37.7-63.9)	0.55 (0.38-0.80)	1.44 (1.10-1.88)	0.38 (0.20-0.72)
	PCT >10[#]	6.0 (2.6-11.5)	86.9 (75.8-94.2)	0.46 (0.18-1.16)	1.08 (0.97-1.20)	0.42 (0.16-1.15)
CAP vs. PJP	**PCT ≥0.1**	95.1 (86.3-99.0)	18.8 (4.0-45.6)	1.17 (0.92-1.49)	0.26 (0.06-1.180	4.46 (0.92-21.84)
	PCT ≥0.25	82.0 (70.0-90.6)	50.0 (24.7-75.3)	1.64 (0.99-2.71)	0.36 (0.17-0.75)	4.55 (1.44-14.43)
	PCT ≥0.5	70.5 (57.4-81.5)	56.2 (29.9-80.2)	1.61 (0.90-2.87)	0.52 (0.29-0.94)	3.07 (1.02-9.26)
	PCT ≥2	49.2 (36.1-62.3)	81.2 (54.4-96.0)	2.62 (0.92-7.51)	0.63 (0.44-0.88)	4.19 (1.15-15.03)
PJP vs. TB	**PCT ≥0.1**	81.2 (54.4-96.0)	3.8 (1.2-8.6)	0.84 (0.67-1.07)	4.99 (1.31-18.94)	0.17 (0.04- 0.71)
	PCT ≥0.25	50.0 (24.7-75.3)	15.8 (10.0-23.1)	0.59 (0.36-0.97)	3.17 (1.69-5.93)	0.19 (0.07-0.54)
	PCT ≥0.5	43.8 (19.8-70.1)	34.6 (26.6-43.3)	0.67 (0.38-1.18)	1.63 (1.00-2.66)	0.41 (0.15-1.14)
	PCT ≥2	18.8 (4.0-45.6)	72.9 (64.5-80.3)	0.69 (0.24-1.99)	1.11 (0.86-1.44)	0.62 (0.18-2.17)

Abbreviations: TB, tuberculosis; CAP, bacterial community acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia; PPV, positive predictive value; NPV, negative predictive value; LR, Likelihood ratio; CI, confidence interval. *Cohort prevalences: TB, 63,3% (95%CI, 56.4%-69.9%); CAP, 29.0% (95% CI, 23.0%-35.7%); PJP, 7.6% (95% CI, 4.4%-12.1%)
[#]Analaysis of PCT category of >10 was only performed in TB vs. CAP infection pairs as no PJP participants had PCT exceeding 10µg/L.

Participants with mixed infection

Analysis of baseline characteristics and index test concentrations for those with mixed infection compared with those with single target infections are summarised in table S3. Analysis of both biomarkers showed wide distributions, overlapping with those of the three mono-infections. Elevated CRP was found in all 38 participants with mixed infections. Both CRP and procalcitonin medians were statistically higher in the mixed infection group compared with the PJP group. 3 participants had procalcitonin concentrations <0.1 µg/L, all of whom had CAP dual infection (two with tuberculosis and the other with PJP). Procalcitonin ≥0.25 µg/L captured 29/37 (78.4) with CAP dual infection, ≥ 0.5 µg/L captured 23/37 (62.2%), and 14 (37.8%) exceeded the ≥2 µg/L cut-off, 3 of whom had concentrations above 10 µg/L.

Discussion

We evaluated the diagnostic accuracy of CRP and procalcitonin for differentiating between the three major infections affecting HIV-infected adult inpatients using a case-referent enrollment study design. Our study was one of only a very few to assess the diagnostic accuracy of CRP and procalcitonin for the three commonest infections in HIV-infected inpatients. We found statistically significant differences in median CRP and procalcitonin concentrations between the three infection groups, but there was marked overlap in distributions. Participants with PJP had lower CRP and procalcitonin concentrations. Procalcitonin and CRP had ROC AUCs of around 0.7 for discriminating PJP from CAP and tuberculosis in pairwise comparisons, indicating moderate discrimination, but both biomarkers performed less well in discriminating CAP from tuberculosis. A CRP cut-off of 147 mg/L had high specificity for discriminating PJP from CAP, and high sensitivity for discriminating PJP from tuberculosis. We found cut-offs with sensitivities of 90% or more for CRP for all three target infection pairs, and for procalcitonin for two target infection pairs (tuberculosis versus PJP and CAP versus PJP), but specificities were much lower than the 70% recommended by WHO for tuberculosis screening tests (20). Our findings suggest that CRP and/or procalcitonin should be explored in the

development of clinical prediction rules in seriously ill HIV-infected patients or in a panel of biomarkers.

Previous studies have demonstrated the diagnostic value of CRP in active tuberculosis case detection in otherwise healthy HIV-infected persons, however higher false positive rates were found in passive case detection (8). Elevated CRP is known to occur in all three of our target infections in individuals with HIV, and our finding that CRP concentrations were highest in CAP, follwed by tuberculosis, and lowest in PJP, is consistent with previous studies (17, 18, 27). We found that diagnostic performance of CRP in our study population was limited by widely overlapping distributions between the three target infections, resulting in reduced utility for inpatient populations where these are the three commonest competing aetiologies. Similar low specificity for discriminating between all three infections was shown in a British case notes review study of HIV-infected adults admitted with respiratory infections (13). Conversely, our findings differed from a previous South African study that reported good discrimintaion of CRP (ROC AUC of 0.87) when comparing participants with pneumococcal community-acquired pneumonia and pulmonary tuberculosis (27). We suspect that the disparity may be attributable to differences in participant selection or to the small sample size in the other South African study. Another study found higher specificity (83%), sensitivity 69%, for combined CRP and IL-8 for discriminating bacerial pneumonia from PJP or mycobateriosis, in a cohort that included hospital-acquired infections and other mycobacterial infections beside tuberculosis (17).

Guidelines for procalcitonin suggest that bacterial infection is present at a concentration of ≥0.25 µg/L (9). Although this cut-off captured 82% of participants with CAP in our study, it also identified 84% of those with tuberculosis and 50% of those with PJP; therefore had limited value in distinguishing CAP from the other two infections. Procalcitonin distribution across the three infections in our cohort, with concentrations highest in CAP and lowest in PJP, were comparable to other studies in HIV-infected individuals (27, 28). However, our findings did not mirror those of two other studies of inpatients with mixed HIV status, both of which found little overlap in procalcitonin concentrations

between any of the three target infections (27, 28). In Schleicher's study, elevated procalcitonin (>0.1 µg/L) was found in all participants with bacterial pneumonia and only 59% of the tuberculosis group, compared with 95% and 96% respectively in our study. Schleicher et al. and Lawn et al. noted a possible link between raised procalcitonin and lower CD4 count (27, 29). In our study we also found a moderate negative association between CD4 count and procalcitonin in patients with tuberculosis (Spearman's rho -0.33, p-value=0.0001). Differing CD4 count medians (107 x 10^9 cells/L in Schleicher's cohort compared with 77 x 10^9 cells/L in ours) may account for higher procalcitonin concentrations in our tuberculosis group.

Mixed infection is a well-recognised limitation in diagnostic accuracy studies. Our study prevalence of 15.3% mixed infection compared to Nyamande's reported 21% (28). In our study, elevated CRP was found in all 38 participants with mixed infection and elevated procalcitonin in 92%. Our participants with mixed infection had higher median concentrations of both CRP and procalcitonin than the participants with tuberculosis and PJP as single infections, but statistical significance was only found when comparing mixed infection to PJP as a single infection. Due to the difficulty in determining the extent of contribution of each infection to biomarker concentrations, the mixed infection group was excluded from diagnostic accuracy analyses.

Our study has a number of limitations. First, reference standards for CAP and PJP did not include microbiological confirmation. Although blood and sputum cultures were carried out to detect bacterial infections, almost all were negative due to prior antibiotic use. However, even if cultures had been taken prior to antibiotics the sensitivity of sputum and blood cultures for CAP is low, and clinical case definitions of CAP are universally used in clinical research. Bronchoalveolar lavage, which is the optimal specimen for diagnosing PJP, was not available at either of our study hospitals. However, we adapted the CDC case definition for PJP, which had a sensitivity and specificity of 85% for diagnosis of PJP when compared with bronchoscopy (30). Furthermore, we found β-D-glucan exceeding 300 pg/L in 13/16 of our participants fulfilling our PJP case definition. β-D-glucan had a sensitivity of 92% and specificity of 78% for the diagnosis of PJP in HIV-infected patients in a systematic review

(31), and 91% sensitivity and 92% specificity for a β-D-glucan cut-off >300 (32). Conversely, elevated β-D-glucan in 11 participants with tuberculosis and one with CAP may represent unrecognized co-infection with PJP. Second, our sample size of participants with PJP was small. Third, CRP and procalcitonin concentrations may have been reduced by antibiotic treatment prior to providing a blood specimen at study enrolment. However, this is unlikely to have had a major effect as almost all of our participants received antibiotics within 24 hours of study enrolment. Study strengths include the use of a robust culture-based reference standard for tuberculosis and that our study participants were recruited from two urban district hospitals that represent a population typical of high HIV and TB burden settings.

In conclusion, CRP and procalcitonin were both found to have limited value in distinguishing between the three common infections due to widely overlapping distributions, particularly between tuberculosis and CAP. Future studies should include a larger sample of participants with PJP definitively diagnosed, as both biomarkers had best diagnostic accuracy for discriminating between PJP and the other two infections in our study. CRP and procalcitonin may have greater diagnostic utility as part of a panel of biomarkers or in clinical prediction rules.

Acknowledgements

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SUPPLEMENTARY DATA

Table S1: Power calculation for sensitivity estimates for each target infection

% CI	95% CI			99% CI		
Sensitivity	**70%**	**80%**	**90%**	**70%**	**80%**	**90%**
TB						
n=133	61.66-77.07	72.07-85.66	84-94.2	69.41-87.17	69.41-87.17	81.56-95.06
n=113	60.91-77.6	71.31-86.04	83.41-94.48	68.38-87.63	68.38-87.63	80.7-95.36
CAP						
n=61	58.11-80.44	68.69-88.37	80.16-95.41	54.06-82.91	64.55-90.15	76.1-96.35
n=52	55.73-80.09	68.10-89.2	79.4-95.82	51.38-82.73	63.58-91	74.9-96.3
PJP						
n=16	44.4-85.84	57- 93.4	64-95.5	37.56-88.94	49.06- 95.12	55.5-97.52
n=14	45.35-88.28	52.41-92.42	68.53-98.73	38.01-91.07	44.37-94.4	59-99.16

Abbreviations: CI: confidence intervals, TB: tuberculosis, CAP: bacterial community-acquired pneumonia, PJP: *Pneumocystis jirovecii* pneumonia.

Table S2: Power calculation to detect C-reactive protein and procalcitonin concentration differences between target disease pairs

Test	Reference mean	Absolute differences across all ranges	Approximate effect size (% difference)	Approximate power (%) with different error probabilities & sample sizes 100% n (TB:133 CAP:61, PJP:16)		85% n (TB:113, CAP:52, PJP:14)	
PCT	**ng/ml (95% CI)**			**0.05**	**0.10**	**0.05**	**0.10**
PTB vs. PJP	4.164	6	92	100	100	100	100
	(1.749-6.579)	2.1	50	88	94	83	91
	1.138	2	48	85	92	79	88
	(0.543-1.734)	0.02	0.009	-	-	-	-
CAP vs. PJP	19.479	19	97	100	99	99	100
	(8.02-30.94)	18	94	99	99	99	100
	1.138	12	62	86	81	81	89
	(0.543-1.734)	10	51	74	84	67	80
		6	78	39	54	35	50
CAP vs. PTB	19.479	29	94	100	100	100	100
	(8.02-30.94)	15	79	96	98	93	97
	4.164	12	62	87	93	81	90
	(1.749-6.579)	11	56	80	89	75	85
		1	0.05	-	15	-	14
CRP	**mg/L (SD)**			**100% 0.05**	**100% 0.10**	**85% 0.05**	**85% 0.10**
PTB vs. PJP	98.7 (48.3)	52	53	99	100	97	99
		36	36	85	92	80	89
	47.0 (49.0)	30	30	72	84	67	80
CAP Vs. PJP	217.0 (16.0)	78	36	100	100	100	100
		35	16	85	93	80	90
	47.0 (49.0)	30	14	75	86	69	82
CAP Vs. PTB	217.0 (16.0)	55	25	100	100	99	100
		35	16	85	93	80	90
	98.7 (48.3)	30	14	75	86	70	82

Abbreviations: CI: confidence intervals, TB: tuberculosis, CAP: bacterial community-acquired pneumonia, PJP: *Pneumocystis jirovecii* pneumonia.

Table S3: Baseline characteristics of 248 participants with a single target infection or mixed infection by infection status

Diagnosis (%)	Total N=248	TB n=133 (54)	CAP n=61 (25)	PJP n=16 (6)	Mixed infection n= 38 (15)	P-value for pairwise comparison*
Median age in yrs. (IQR)	34.7 (29.1-41.2)	34.7 (29.1-40.1)	35.1 (29.4-40.0)	36.9 (28.8-41.2)	34.5 (29.9-43.8)	TB vs. MI=0.66, CAP vs. MI=0.74, PJP vs. MI=0.91
Sex: female n(%)	168 (68)	84 (63)	44(72)	11(69)	29(76)	TB vs. MI=0.13, CAP vs. MI=0.65 PJP vs. MI= 0.57
Cotrimoxazole prophylaxis	73 (29)	38(29)	18(30)	4(25)	13(34)	TB vs. MI=0.50, CAP vs. MI=0.63 PJP vs. MI= 0.75#
Antiretroviral therapy n (%)	93 (38)	43(32)	29(48)	4(25)	17(45)	TB vs. MI=0.16, CAP vs. MI=0.79 PJP vs. MI=0.23#
Median $CD4^+$ count, cells/µL (IQR)	94 (36-210)	77 (35-162)	199.5 (78.5-287)	35 (11.5-80.5)	79.5 (23-202)	TB vs. MI=0.9, CAP vs. MI=0.004 PJP vs. MI=0.17
Median WCC $x10^9$/L (IQR)	8.9 (5.8-12.9)	7.3 (5.2-10.2)	12.3 (8.4-20.0)	8.2 (6.2-10.7)	10.3 (7.2-14.8)	TB vs. MI=0.005, CAP vs. MI=0.10 PJP vs. MI=0.16
Median Hb g/dl (IQR)	9.4 (7.7-10.9)	8.6 (7.4-10.1)	10.4 (8.8-12)	11.25 (9.7-12.2)	9.4 (7.8-11.6)	TB vs. MI=0.05, CAP vs. MI=0.14 PJP vs. MI=0.05
Median CRP mg/L (IQR)	149.5 (92-223.5)	141 (97-203)	193 (108-264)	106.5 (79.5-131.5)	184.5 (82-223)	TB vs. MI: 0.52, CAP vs. MI: 0.25 PJP vs. MI: 0.03
Median PCT µg/L (IQR)	0.8 (0.3-3.2)	0.7 (0.4-2.1)	2.0 (0.4-5.2)	0.2 (0.1-1.3)	0.7 (0.3-4.2)	TB vs. MI: 0.96, CAP vs. MI: 0.26 PJP vs. MI: 0.09
PCT≥0.25 µg/L n(%)	200 (80.7)	112 (84.2)	50 (82.0)	8 (50.0)	30 (79.0)	PTB vs. MI: 0.45, CAP vs. MI: 0.71 PJP vs. MI: 0.03
WHO danger signs[1]:						
Pulse rate> 120beats/min[1]	199 (80)	106 (80)	51 (84)	9 (56)	33(87)	TB vs. MI=0.32, CAP vs. MI=0.66 PJP vs. MI= 0.01
Respiratory rate>30/min[1]	162(65)	83 (62)	38 (62)	16 (100)	25(66)	TB vs. MI=0.70, CAP vs. MI=0.73 PJP vs. MI=0.006#
Temperature >39°C[1]	38 (15)	20 (15)	10 (16)	1 (6)	7 (18)	TB vs. MI=0.61, CAP vs. MI=0.80 PJP vs. MI= 0.25
Unable to walk unaided[1]	140 (56)	88 (67)	23 (38)	8 (50)	21(55)	TB vs. MI=0.20, CAP vs. MI=0.10 PJP vs. MI=0.72

Abbreviations: TB, tuberculosis; CAP, bacterial community-acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia; MI: mixed infection; Hb: haemoglobin; WCC: white cell count; CRP, C-reactive protein; PCT, procalcitonin

*Hypothesis tests- Wilcoxon-Mann-Whitney test for continuous data; Chi-square test for categorical data. #Fisher's exact test where 1 or more cells <5

[1] Danger signs based on WHO algorithm for diagnosis of TB in seriously ill patients.

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These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Publication Info section agrees to be named.

> Do not include funding sources in the Publication Info or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL. Read the Preprint Policy.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

> Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. *Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.*
Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and Tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

Read the guidelines for figures.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”).
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., “Table 1”) and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS licenses and copyright policy also applies to striking images.

Additional Information Requested at Submission

Funding Statement

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission**.**

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

Read our policy on disclosure of funding sources.

Competing Interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into “parts.” Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

PLOS does support authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors choosing bioRxiv may now concurrently submit directly to select PLOS journals through bioRxiv’s direct transfer to journal service.

Read our policies on related manuscripts and preprint servers.

Guidelines for Specific Study Types

Human subjects research

All research involving human participants must have been approved by the authors’ Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

> *A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.*

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF), including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention (“death as an endpoint”), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How “Humane” Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.
- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

> *All necessary permits were obtained for the described study, which complied with all relevant regulations.*

If no permits were required, please include the following statement:

> *No permits were required for the described study, which complied with all relevant regulations.*

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document

- Flow diagram: PDF or Word document

Authors must also state in their “Methods” section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select “Research Article” as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants’ experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

> We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation

functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

Utility
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability
If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a databse, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials and software.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> ***Anochetus boltoni*** *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

> The electronic edition of this article conforms to the requirements of the amended International Code of Zoological

Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> **Solanum aspersum** S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> ***Hymenogaster huthii.*** Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

> The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
>
> In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

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